Study of the Wheat Resistance Gene *Lr34* after its Transfer into Heterologous Crop Species

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Summary

Because of global population growth, agricultural production needs to double by 2050. Plant diseases are responsible for ~10% loss of global crop production. The most sustainable strategy to reduce yield losses caused by pathogens is through exploiting disease resistance mechanisms. *Lr34* is a wheat gene that confers durable and partial resistance against multiple fungal pathogens. The resistant *Lr34* allele evolved after wheat domestication and a *Lr34*-like disease resistance has not been described in other globally important cereals such as rice, maize or sorghum. *Lr34* encodes a full-size ATP binding cassette (ABC) –transporter.

The objective of this PhD thesis was to understand the mechanism of *Lr34*-mediated durable resistance through its functional transfer into the heterologous species rice and maize, which are among the most consumed cereals. Transgenic rice and maize lines showed an increased resistance against the fungal diseases rice blast (*Magnaporthe oryzae*), maize common rust (*Puccinia sorghi*) and northern corn leaf blight (*Exserohilum turcicum*), respectively. These results show that the *Lr34*-resistance can be functionally transferred into different cereals, indicating that the *Lr34* substrate is conserved among these species.

A second part of the PhD focused on transcriptional changes in pathogens that grew on *Lr34*-expressing plants. This study showed that *Lr34* does not modulate gene expression patterns in pathogens. This absence of differences from the pathogen side could provide an explanation for the durability of *Lr34*.

This thesis highlighted the conserved mechanism of *Lr34* among different cereal species by its functional transfer.

In the future, *Lr34* could be a very useful tool in order to improve disease resistance in crop of agronomical importance such as rice or maize and would provide a great agricultural advantage.

Zusammenfassung

Aufgrund des weltweiten Wachstums der Bevölkerung, muss die landwirtschaftliche Produktion bis 2050 verdoppelt werden. Pflanzenkrankheiten sind für ~10 % des globalen Ernteverlusts verantwortlich. Daher ist die probateste Strategie Ernteverluste durch Pathogene zu vermindern, die Ausnutzung von Krankheitsresistenzmechanismen. *Lr34* ist ein Weizengen, welches dauerhafte und partielle Resistenz gegen verschiedene Pilzpathogene verleiht. Das resistente *Lr34*-Allel evolvierte nach der Domestikation von Weizen. Eine vergleichbare Krankheitsresistenz wurde in anderen global wichtigen Getreiden, wie Reis, Mais oder Hirse, nicht beschrieben. *Lr34* kodiert für einen full-length ATP binding cassette (ABC)-Transporter.

Das Ziel dieser PhD Thesis war, den Mechanismus der *Lr34*-vermittelten dauerhaften Resistenz mittels funktionellem Transfer des Gens in die heterologen Spezien Reis und Mais, zwei der am meist konsumierten Getreide, zu verstehen. Transgene Reis- und Maislinien zeigten eine erhöhte Resistenz gegen die Pilzpathogene Reisblast (*Magnaporthe oryzae*), beziehungsweise gegen Maisrost (*Puccinia sorghi*) und Turcicum-Blattflecken (*Exserohilum turcicum*). Dass die *Lr34*-Resistenz in verschiedene Getreide funktionell transferiert werden kann, indiziert, dass das Substrat von *Lr34* in diesen Spezien konserviert ist.

Ein zweiter Teil des PhDs fokussierte sich auf transkriptionelle Veränderungen in Pathogenen, die auf *Lr34*-exprimierenden Pflanzen gewachsen sind. Diese Studie zeigte, dass *Lr34* die Expressionsmuster von Pathogenen nicht moduliert. Die Abwesenheit von Differenzen auf Seiten des Pathogens könnte eine Erklärung für die Dauerhaftigkeit von *Lr34* liefern.

Diese Thesis hebt den konservierten Mechanismus von *Lr34* zwischen verschiedenen Spezien, durch dessen funktionellen Transfer, hervor.

Lr34 könnte sich in Zukunft als nützliches Werkzeug zur Verbesserung von Krankheitsresistenz in agronomisch wichtigen Getreiden, wie Reis und Mais, erweisen und einen grossen agronomischen Vorteil liefern.

Chapter A

General Introduction

(1) The three most important cereal species: maize, rice and wheat

Maize, rice and wheat are the most important cereal species in the world, with 1,016 million tons, 745 million tons and 713 million tons produced in 2013 (FAO, 2016), respectively. These crops contribute to human food as well as animal feeding and were domesticated about 10,000 years ago (Garris *et al*. 2005; Sang 2009). In the mid-1960's, during the Green Revolution, new high-yielding varieties were successfully released (Evenson and Goldin 2003) leading to a massive production increase over the years (FAOstat) (Figure 1.).

The United States are the biggest maize producer before China and Brazil (FAOstat) and the wheat production is led by the European-Union before China, India and Russia (FAOstat). In contrast to maize and wheat, the rice production is mainly located in Asia where more than 90% of the global world production is grown (Sharif *et al.* 2014). Wheat and rice are mainly grown for human consumption compared to the maize production which is also used for animal feeding and for the production of a number of industrial products such as starch, oil, ethanol and malt (Chaudhary *et al*. 2014).

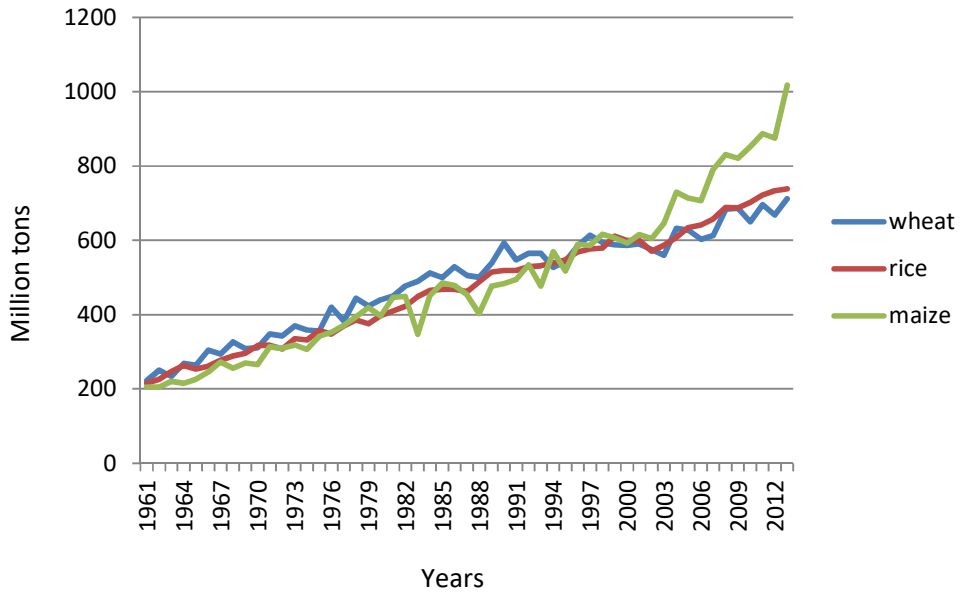

Figure 1. World production in million tons of the three major cereals wheat, rice and maize, between 1961 and 2014. Adapted from FAOstat.

(2) Globally important fungal disease of maize, rice and wheat

2.1 – The biotrophic pathogens

Among the biotrophic pathogens, rust fungi are the most devastating and economically important since they cause significant yield losses every year. As all obligate biotrophs, the different rusts fungi depend on living plant tissue to grow and complete their life cycle. Rusts are a diverse group of plant pathogens, all belonging to the Pucciniales, Basidiomycota group. This group is composed of more than 700 species (Duplessis *et al.* 2011) which grow on numerous crop species, including wheat, barley and maize. Leaf rust (*Puccinia triticina / Puccinia hordei*), stripe rust (*Puccinia striiformis f.sp tritici or hordei)* and stem rust *(Puccinia graminis f.sp tritici or hordei)* are the three rusts infecting wheat and barley. Several rust diseases occur in maize and one of the best known is the maize common rust, caused by the *Puccinia sorghi* pathogen (Pryor 1994).

Leaf rust is the most common of the three wheat rusts and yield losses can be as high as 30% to 50% in years of severe infection (Boydom *et al*. 2013). The symptoms of leaf rust usually appear after 7 to 10 days and are characterized by orange-coloured urediniospores produced in pustules occurring on the leaf surface (Figure 2.).

Rice is the only intensively grown crop which is not affected by rusts and the molecular mechanism of its immunity is still unknown (Ayliffe *et al*. 2011). All rusts require an alternate host to complete their sexual life cycle. The predominantly alternate host species of wheat and barley leaf rusts are respectively *Thalictrum speciosissimum* and *Ornithogalum spp.* (Anikster, 1982; Bolton *et al*. 2008) whereas common barberry (*Berberis vulgaris)* is the alternate host of stripe and stem rusts (Jin, 2011). The alternate host of the maize common rust is *Oxalis spp.,* a perennial and widespread weed (Guerra *et al.* 2016).

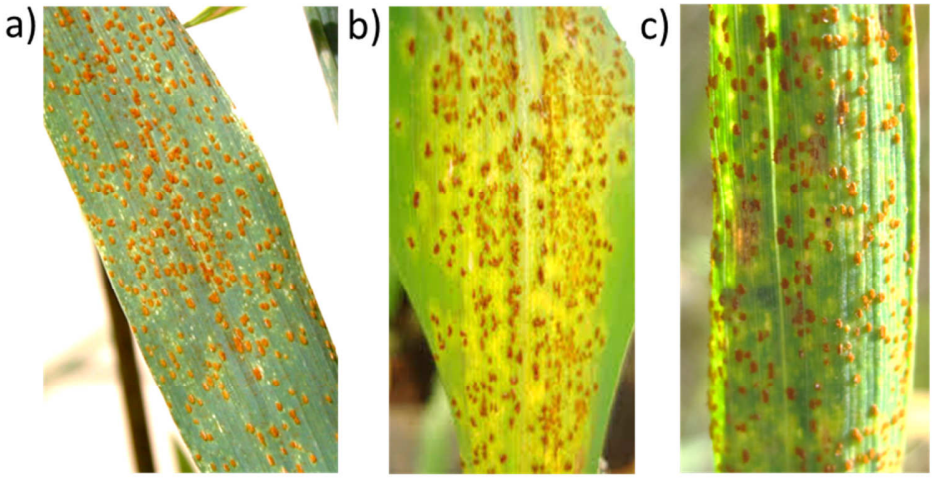

Figure 2. Leaf rust symptoms on a) wheat (*Puccinia triticina*) b) maize (*Puccinia sorghi*) c) barley (*Puccinia hordei*).
https://www.agric.wa.gov.au/mycrop/diagnosing-leaf-rust-wheat
http://www.apsnet.org/publications/imageresources/Pages/fi00247.aspx
http://tolweb.org/Pucciniomycetes/51246

Powdery mildew is a fungal disease caused by another obligate biotroph pathogen and affects a large variety of crop species: wheat and barley are the hosts of *Blumeria graminis f.sp. tritici* and *Blumeria graminis f.sp. hordei* respectively. Mildews belong to the order of *Erysiphales*, *Ascomycota*, a phylum distinct from *Basidiomycota*. In contrast to rust, they do not require an alternate host for the life cycle completion (Heffer *et al*. 2006). Symptoms, which are white colonies formed from the epiphytic mycelium, occur 5 to 7 days after infection and can cause up to 30% of yield losses (Parashivu *et al*. 2014) (Figure 3.).

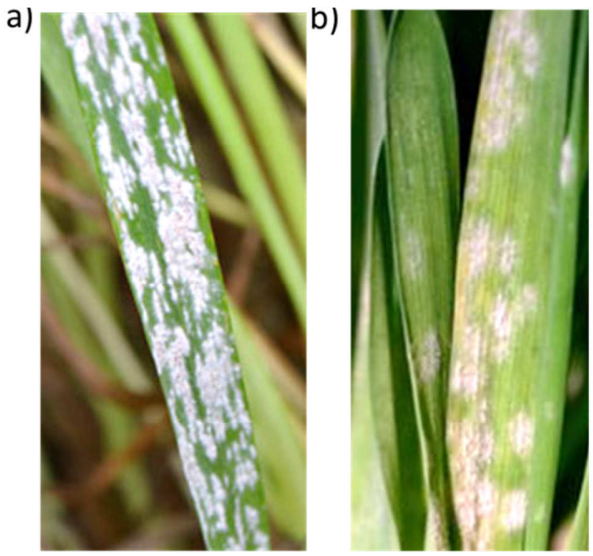

Figure 3. Powdery mildew symptoms on a) wheat b) barley.
http://cropwatch.unl.edu/wheat-disease-update-scout-fields-early-disease-detection
http://www.ipk-gatersleben.de/en/breeding-research/pathogen-stress-genomics/projects/

Fungal pathogens causing rust and powdery mildew diseases share similar characteristics of the obligate biotroph infection process such as the formation of a feeding structure called haustorium once the pathogen is established in the plant cells (Figure 4.a) (Song *et al.* 2011; Pederson et al. 2012; Haugaard *et al*. 2002; Zabka *et al*. 2008). The haustorium allows the fungus to obtain nutrients from the plant but also to secrete small molecules called effectors which are key players in the manipulation of physiological and immune responses of host cells (Garnica *et al.* 2014). Hemi-biotrophic fungi have similar needs for nutrient uptake from the host but use a different acquisition strategy.

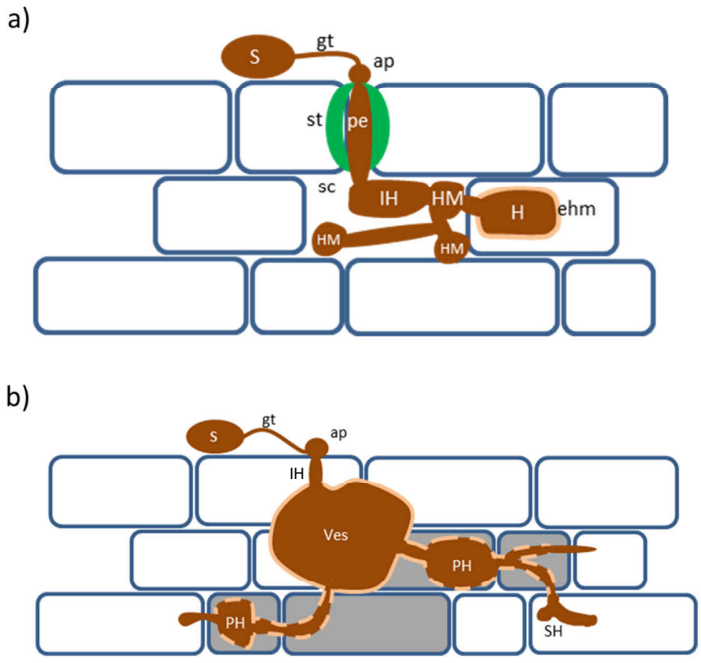

Figure 4. Fungal infection structures a) biotrophic fungi (leaf rust) b) hemi-biotrophic fungi (rice blast). S: spore, gt: germ tube, ap: appressorium, pe: penetration peg, st: stomata, sc: substomatal cavity, Ves: vesicle, IH: invasive hyphae, HM: haustorial mother cell, H: haustorium, ehm: extrahaustorial matrix, SH: secondary hyphae, PH: primary hyphae. Adapted from Mendgen and Hahn (2002).

2.2 – The hemi-biotrophic pathogens

Rice blast, caused by *Magnaporthe oryzae* is the major fungal disease of rice and can cause more than 60% of yield loss, even up to 100% in severe epidemics (Kihoro *et al.* 2013). Rice blast is also known to infect other cereal species such as wheat and barley (Ulferts *et al*. 2015).

All hemi-biotrophic fungi combine two lifestyles, the biotrophic and the necrotophic one. The infection process starts with spore germination and appressorium formation. Rice blast penetrates the leaf directly through the cuticle and then forms a feeding structure called vesicle, similar to the haustorium of rusts and mildew. This vesicle is produced by a differentiation of the invasive hyphae (Wilson *et al*. 2012; Kankanala *et al.* 2007) and corresponds to the biotrophic phase of the infection. After two days, rice blast switches to a necrotrophic phase leading to the destruction of the host tissue (Marcel *et al.* 2010; Levy and Cohen; 1983) (Figure 4.b). Necrotic symptoms occur 7 days after infection and are characterized by their diamond-shape lesion with a grey or white center (Srivastava *et al*. 2014) (Figure 5.a).

The maize northern corn leaf blight disease, caused by the fungal pathogen *Exserohilum turcicum* has a hemi-biotrophic lifestyle with a similar infection mechanism as rice blast. This foliar fungal disease is very important, especially in the United States which is the biggest maize producer, where it can cause more than 50% of grain yield losses (Van Inghelandt *et al*. 2012). The symptoms are small light-green lesions to grayish spots that appear approximately 8 days post infection, leading to long gray colored cigar-shaped lesion on the leaves (Figure 5.b).

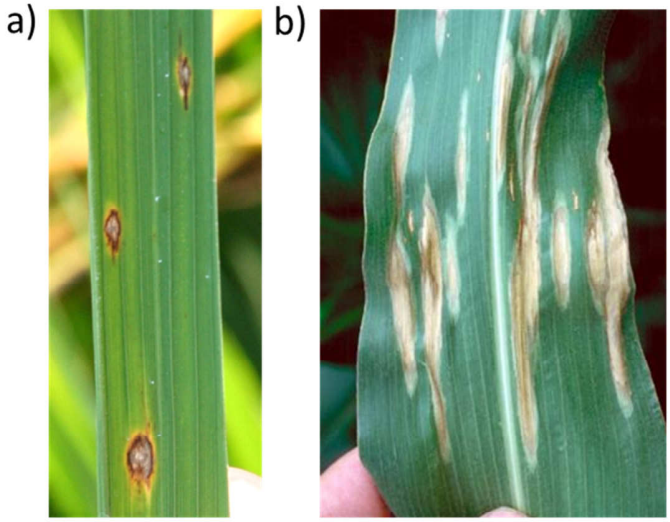

Figure 5. Symptoms of hemi-biotrophic diseases of a) rice blast on rice and b) northern corn leaf blight on maize.

http://ucanr.edu/blogs/riceblog/index.cfm?tagname=diseases

http://www.ent.iastate.edu/imagegal/plantpath/corn/northleafblight/ncorn_leaf_blight_0796_02.html

(3) Disease resistance genes and pathogen management

3.1 – Crop breeding methods

Plant breeding already started about 10,000 years ago with crop (wheat, maize and rice) domestication (Eckard 2010), which marked an important turn in human civilization. The Green Revolution, which took place in the mid-20th century, marked an important milestone in plant breeding, when traditional low yielding cultivars were replaced by high-yield modern varieties. The Green Revolution was also characterized by the introduction of pesticides, fungicides and fertilizer into agriculture. Later on, genomics tools have contributed to the identification of genes linked to traits of interest, facilitating the development of new varieties (Khush 2001).

Genes coding for proteins resulting in traits of interest can be identified thanks to genetic mapping populations and the use of genetic and physical markers such as single nucleotide polymorphisms (SNP), simple sequence repeats (SSRs) or expressed sequence tags (ESTs) markers. These genomics approaches are based on genomic DNA analysis and became easier and faster to use due to the progress of next generation sequencing technologies, bioinformatics as well as crop genome database improvement. Marker-assisted-selection (MAS) is a useful tool for crop breeding which allows to determine the presence of genes underlying traits of interest by DNA amplification and sequencing (Collard and Mackill 2008). A powerful combination between genomics and phenotyping data is based on genome-wide association studies (GWAS) which consist of an examination of a genome-wide set of genetic variants in different individuals to associate a phenotypic trait of interest, such as disease resistance, to a SNP in the genome (George and Cavanagh 2015).

3.2 – Resistance genes in plant breeding

Plants are constantly attacked by pathogens and evolved a plethora of resistance mechanism to protect themselves. Resistance can be classified based on several criteria, including the specificity, the durability, the level of the resistance and the inheritance.

Race-specific resistance works against one or several races of a given pathogen. Most of the time, this resistance is strong or complete and also called qualitative resistance. In many cases,

qualitative resistance has been associated with R-gene resistance (based on a single major gene) and a gene for gene interaction where the pathogen effectors are recognized by the host and lead to defense activation (Flor, 1971).

R genes have been extensively used in breeding but have been shown to be frequently non-durable because of a rapid pathogen adaptation. In the gene for gene interaction, the resistance is provided by the recognition of pathogen effectors by the R gene, leading to effector-triggered immunity (ETI) which is the activation of the defense mechanisms of the host and is most of the time associated with hypersensitive response (HR).

In contrast to race specific resistance genes, the race non-specific resistance genes are usually more durable thanks to different resistance mechanisms. The resistance provided by race non-specific resistance genes is, in most of the cases, quantitative but efficient against all the races of one or even several pathogens and efficient at adult stage (Lindhout, 2002). This partial resistance acts by delaying/or reducing growth of the pathogen compared to a susceptible plant (Ribeiro Do Vale *et al.* 2001).

3.3 – Examples of resistance genes

In wheat and other crop species more than hundred R genes have been genetically described and used in breeding programs (Singla and Krattinger, 2016). As examples for cloned genes, *Pm3 (*Yahiaoui *et al*. 2004)*,* which confers powdery mildew resistance as well as *Lr10* (Feuillet *et al.* 2003) and *Lr21 (*Huang *et al.* 2003)*,* which are leaf rust resistance genes, are all R genes encoding nucleotide-binding domain and leucine-rich repeat containing (NLR) family class of proteins. In rice, the Pi-ta R-gene, which provide rice blast resistance also encodes for an NLR protein (Huang *et al.* 2008).

Several race nonspecific resistance genes have been identified such as the barley *mlo* gene (Wang *et al.* 2014) which confers recessive powdery mildew resistance and encodes a plant-specific protein structurally related to metazoan G-protein coupled receptors (GPCRs), harbouring seven transmembrane domains and a calmodulin-binding domain that is likely implicated in the perception of calcium-dependent stimuli (Iovieno *et al.* 2015). The wheat *Sr2* (Mago *et al. 201*1) gene provides broad-spectrum resistance against stripe rust, but its molecular identity and function remains unknown. The wheat *Yr36* gene, which provides a race nonspecific resistance

against stripe rust codes for a protein having a kinase and a putative START lipid-binding domain (Fu *et al.* 2009). The *Pi21* rice gene codes for a small proline-rich protein and provides race nonspecific resistance against rice blast (Fukuoka *et al*. 2009).

To date, only three genes have been identified as multi-pathogen quantitative resistance genes and they are all unique to wheat, called *Lr34*, *Lr67* and *Lr46* respectively. *Lr34* has been cloned in 2009 (Krattinger *et al*. 2009) and codes for a putative ATP-binding cassette (ABC) transporter whereas *Lr67* encodes a predicted hexose transporter (Moore *et al*. 2015).

(4) The *Lr34* multi-pathogen resistance gene

4.1 – Specificity of *Lr34*

Lr34 has been extensively used in wheat breeding during the last century and was introduced in more than 50% of all the wheat cultivars grown in major breeding programs (Hoisington *et al*. 1999). *Lr34* provides partial resistance against the most important fungal pathogens: leaf rust, stripe rust, stem rust and powdery mildew. No pathogen adaptation has been observed so far showing the durability of this gene (Kolmer *et al.* 2008). *Lr34* in wheat is an adult plant resistance gene (APR), which is associated with a characteristic leaf tip necrosis (LTN) phenotype. LTN is a senescence-like process occurring at the tip of the leaf. Natural *Lr34*-like resistance, i.e. broad spectrum, multi-pathogens and durable resistance, is unique to bread wheat. This kind of resistance is a real advantage for crop breeding and *Lr34* has been transferred to other cereal crops such as barley (Risk *et al*. 2013), rice (Krattinger *et al.* 2016) and maize (Sucher *et al*. 2016). The functional transfer of *Lr34* to these crops is described in this thesis and resulted in resistance against rice blast (Krattinger *et al.* 2016), maize common rust and northern corn leaf blight (Sucher *et al*. 2016), all important fungal pathogens. Interestingly, in these different transgenic *Lr34* lines, the resistance is already effective at tne seedling stage compared to wheat where *Lr34* is only expressed at adult stage.

4.2 – The two *Lr34* alleles: susceptible and resistant

The *Lr34* wheat gene is located on chromosome 7D and encodes a putative ABC-transporter of the subfamily G (Lagudah *et al*. 2009). ABCG proteins can transport various substrates accross cellular membranes (Yu *et al.* 2015). Two predominant haplotypes of the *Lr34* gene have been reported in the wheat gene pool, a resistant (*Lr34res*) and a susceptible (*Lr34sus*) one, which differ by only two amino acid polymorphisms in exon 11 and exon 12 (Figure 6.) (Lagudah *et al*. 2009; Krattinger *et al.* 2009). These two amino acid changes occur in the first transmembrane domain of the ABC-transporter protein and result in a modification of the protein structure which can lead to changes in protein stability or in protein function. These structural changes could then have an influence on the substrate specificity, substrate affinity or cellular localization. The first sequence polymorphism is a phenylalanine (TTC) deletion in exon eleven and the second polymorphism is a base change (C to T) in the exon 12 which causes the conversion of a tyrosine (in the susceptible variant) to a histidine (in the resistant variant) (Krattinger *et al.* 2006; Lagudah *et al*. 2009). Among these sequence changes between the susceptible and the resistant alleles, the phenylalanine deletion alone has been shown to be sufficient to confer the LTN as well as the resistance phenotype in barley (Chauhan *et al.* 2015).

Wheat transcriptome analysis performed on wheat *Lr34* resistant plants showed that most of the up-regulated genes were associated with abscisic acid (ABA) inducibility and drought stress (Hulbert e*t al.* 2007). In *Lr34* transgenic barley, transcriptomic analysis showed that genes from multiple pathways, contributing to basal and inducible resistance were up-regulated in seedlings and mature leaves (Chauhan *et al.* 2015).

The role of ABA in the *Lr34* mechanism was also shown by Krattinger *et al.* (unpublished). *Lr34* has been shown to modulate the ABA fluxes in the plant. The exact transport mechanism and its relation with the resistance, as well as the function of the susceptible *Lr34* allele remain unknown. Furthermore, we cannot exclude than other substrates could be transported by LR34res, as it was already shown that some ABC transporters can transport more than one substrate (van der Heide and Poolman 2002).

Lr34res is unique to wheat but orthologous genes of the susceptible allele of *Lr34* have been identified in rice (*OsABCG50,* which is 86% identical on protein level) and sorghum (*Sb01g016775*, with 72 % identity on protein level) but not in maize or in barley. All these genes are functionally

uncharacterized so far and there is no evidence that they could be involved in any kind of resistance.

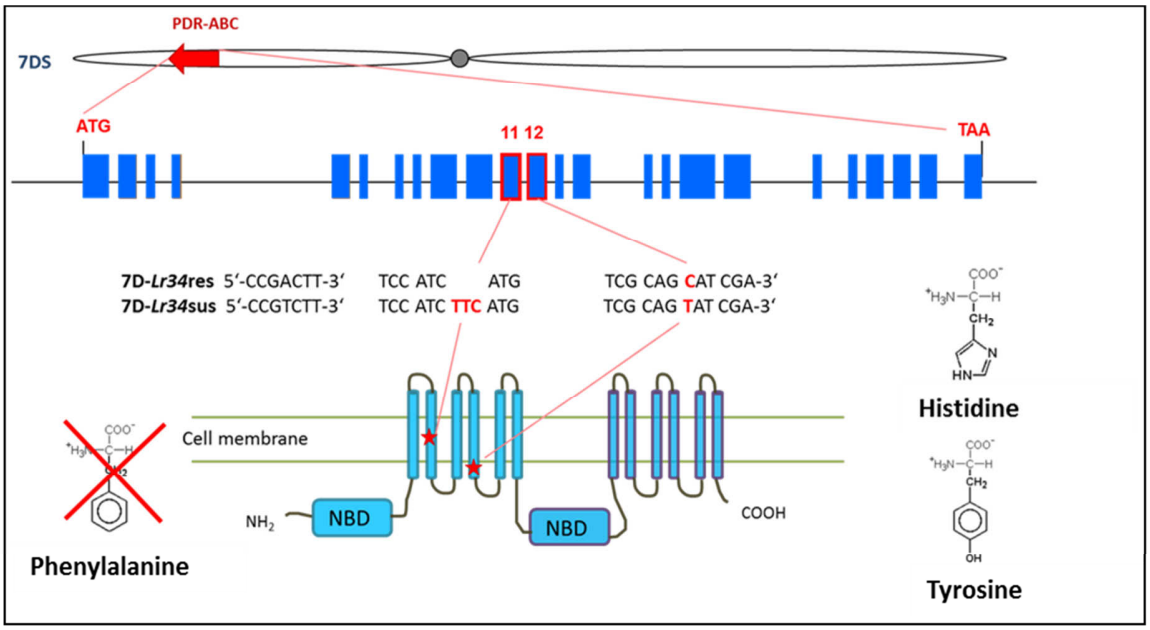

Figure 6. Allelic versions of the wheat gene *Lr34.*

(5) Aim of the thesis

The aim of the thesis was to study the resistance mechanism of *Lr34* by its functional transfer into other crops of agronomical interest, in order to have a better understanding of durable and multi-pathogen broad-spectrum resistance. In chapters (B) and (C) we used the heterologous crop species rice and maize as tools. In chapter (B) we show the functional transfer of *Lr34res* into rice and the resistance it provided against the hemi-biotrophic fungus rice blast and in the chapter (C) the resistance provided against maize common rust and northern corn leaf blight in the transgenic *Lr34res* maize plants. The study of pathogens is essential to have a better understanding of the disease resistance mechanisms. In chapter (D), we performed transcriptomics analysis of the wheat leaf rust and the barley powdery mildew growing on *LR34* resistant and susceptible plants.

This study did not reveal differences in the expression pattern suggesting that *Lr34* does not modulate the pathogen gene expression profile.

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Chapter B

The wheat durable, multi-pathogen resistance gene *Lr34* confers partial blast resistance in rice

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Summary

The wheat gene *Lr34* confers durable and partial field resistance against the obligate biotrophic, pathogenic rust fungi and powdery mildew in adult wheat plants. The resistant *Lr34* allele evolved after wheat domestication through two gain-of-function mutations in an ABC transporter gene. An *Lr34*-like fungal disease resistance with a similar broad-spectrum specificity and durability has not been described in other cereals. Here, we transformed the resistant *Lr34* allele into the *japonica* rice cultivar Nipponbare. Transgenic rice plants expressing *Lr34* showed increased resistance against multiple isolates of the hemibiotrophic pathogen *Magnaporthe oryzae,* the causal agent of rice blast disease. Host cell invasion during the biotrophic growth phase of rice blast was delayed in *Lr34*-expressiong rice plants, resulting in smaller necrotic lesions on leaves. Lines with *Lr34* also developed a typical, senescence-based leaf tip necrosis (LTN) phenotype. Development of LTN during early seedling growth had a negative impact on formation of axillary shoots and spikelets in some transgenic lines. One transgenic line developed LTN only at adult plant stage which was correlated with lower *Lr34* expression levels at seedling stage. This line showed normal tiller formation and more importantly, disease resistance in this particular line was not compromised. Interestingly, *Lr34* in rice is effective against a hemibiotrophic pathogen with a lifestyle and infection strategy that is different from obligate biotrophic rusts and mildew fungi. *Lr34* might therefore be used as a source in rice breeding to improve broad-spectrum disease resistance against the most devastating fungal disease of rice.

(1) Introduction

Fungal plant diseases are a serious threat to cereal production. Breeding for effective and durable field resistance is the most sustainable strategy to reduce yield losses caused by pathogenic fungi. Fungal disease resistance genes in cereals can be broadly classified into three categories based on their specificity and durability. The first group contains genes that confer race-specific resistance against some but not all races of a particular pathogen. These so called *R* genes often encode intracellular immune receptor proteins belonging to the nucleotide binding site-leucine-rich repeat family (NBS-LRR). R proteins directly or indirectly perceive pathogen-derived virulence effectors that are secreted into the cytoplasm of host cells in order to suppress basal immunity. This interaction triggers a strong resistance reaction called hypersensitive response (HR) that often results in death of the infected cell (Dodds and Rathjen, 2010). Mutations in pathogen effectors that escape recognition by plant NBS-LRR immune receptors have a strong selective advantage. For this reason, disease resistance based on single *R* genes is often not durable in the field and breakdown of *R* gene-based resistance is frequently observed in cereals (Park et al., 2002; Pretorius et al., 2000; Wilson and Talbot, 2009). The second group of cereal disease resistance genes confers race non-specific resistance against all races of a particular pathogen. Examples include the wheat stripe rust resistance gene *Yr36* that encodes a protein with a kinase domain fused to a START lipid-binding domain (Fu *et al.,* 2009), the recessive rice blast resistance gene *pi21* that encodes a small proline-rich protein (Fukuoka *et al.,* 2009), or the recessive barley powdery mildew resistance gene *mlo* that encodes an integral membrane protein (Buschges *et al.,* 1997). The third group contains genes that confer race non-specific resistance against multiple fungal pathogens simultaneously. Only three of these broad-spectrum, multi-pathogen resistance genes have been described in cereals to date. The three wheat genes *Lr34* (same as *Yr18/Sr57/Pm38*), *Lr46* (=*Yr29/Sr58/Pm39*) and *Lr67* (=*Yr46/Sr55/Pm46*) confer partial resistance at the adult plant stage against all races of the obligate biotrophic fungi leaf rust (*Puccinia triticina*), stripe rust (*Puccinia striiformis* f.sp. *tritici*), stem rust (*Puccinia graminis* f.sp. *tritici*) and powdery mildew (*Blumeria graminis* f.sp. *tritici*) (Ellis et al., 2014). To our knowledge, this resistance-type has so far not been reported in cereals other than wheat.

The majority of cereal disease resistance genes that have been identified and cloned so far belong to the race-specific NBS-LRR gene family. In contrast to *R* genes, resistance based on race non-specific genes is usually more durable in the field (Lagudah, 2011). Of the three wheat multi-pathogen resistance genes, only *Lr34* has been cloned to date. It encodes a full-size ATP-binding cassette (ABC) transporter belonging to the ABCG (formerly PDR) subfamily (Krattinger et al., 2009; Risk et al., 2012). Full-size ABCG transporter proteins share a conserved domain structure consisting of two nucleotide-binding domains and two transmembrane domains. These proteins use the energy derived from ATP hydrolysis to shuttle various substrates across cellular membranes (Rea, 2007). *Lr34* has been used in wheat breeding since the early 20th century and no increased pathogen adaptation has been observed (Kolmer et al., 2008). The gene delays pathogen development by prolonging the latency period and decreasing infection frequency (Rubiales and Niks, 1995). This partial disease resistance phenotype is also referred to as slow-rusting or slow-mildewing. So far, only one resistance-conferring *Lr34* allele, named *Lr34res* has been identified in the wheat gene pool. This particular allele emerged after wheat domestication about 8,000 years ago and is the result of two gain-of-function mutations that are critical for disease resistance (Dakouri et al., 2010; Krattinger et al., 2013; Krattinger et al., 2011). These two mutations resulted in a deletion of a phenylalanine residue and a conversion of a highly conserved tyrosine to histidine in the resistant LR34 ABC transporter version. Both amino acid changes are located in the first transmembrane domain of the transporter. *Lr34res* is only found in cultivated bread wheat (*Triticum aestivum* L.) but not in wild wheat progenitors which is in agreement with the emergence of this allele after domestication. Most wild wheat progenitors and susceptible wheat cultivars carry an expressed *Lr34* allele called *Lr34sus* with a complete open reading frame encoding a putatively functional ABC transporter. The function of *Lr34sus* however is not yet known. Furthermore, rice and sorghum carry an expressed *Lr34* ortholog but only the susceptible *Lr34* haplotype for the two critical amino acid polymorphisms was found in these orthologous *Lr34* genes in rice and sorghum (Krattinger *et al.* 2013). *Lr34res* is associated with a senescence-like process called leaf tip necrosis (Singh, 1992). The development of LTN in adult wheat plants is dependent on the genetic background and environmental conditions. Risk et al. (2013) showed that *Lr34res* is functionally transferable to the close wheat relative barley (*Hordeum vulgare* L.) where *Lr34res* but not *Lr34sus* conferred partial resistance against the barley-specific diseases barley leaf rust (*Puccinia hordei*) and barley powdery mildew (*Blumeria graminis* f.sp. *hordei*). In

contrast to wheat, where *Lr34res* is ineffective at seedling stage under normal temperatures, *Lr34res* conferred seedling resistance in barley. However, *Lr34res*-barley also developed a severe LTN during the seedling stage resulting in poor plant growth and reduced seed production.

Rice (*Oryza sativa* L.) is one of the most widely grown crops and provides a daily staple for more than half of the world's population. In this study, we transformed *Lr34res* into the *japonica* rice cultivar Nipponbare. *Lr34res*-carrying rice plants showed increased resistance against the hemibiotrophic fungal pathogen *Magnaporthe oryzae,* the causal agent of rice blast disease. These results show that a broad-spectrum disease resistance gene can be functional across different cereal species against diverse biotrophic and hemibiotrophic pathogens.

(2) Results

2.1 – Transgenic expression of *Lr34res* confers partial rice blast resistance

A construct containing the genomic sequence of the wheat resistant *Lr34res* allele under its native promoter (Risk *et al.*, 2013) was transformed into the *japonica* rice cultivar Nipponbare. Homozygous lines of five independent transgenic events were selected based on the presence and expression of *Lr34res*. Southern Blot analysis indicated that three events (numbers 8, 11 and 19) carried single T-DNA insertions, one event (16) had two co-segregating insertions and one event (5) showed multiple co-segregating copies (Figure S1). In wheat and barley, *Lr34res* confers resistance against obligate biotrophic pathogens belonging to the cereal rusts (*Puccinia* spp.) and cereal powdery mildews (*Blumeria graminis*; Krattinger et al., 2009; Risk et al., 2013). Rice however is immune to all powdery mildew and rust fungi (Ayliffe et al., 2011; Cheng et al., 2014). The five transgenic events were subjected to infection experiments with the hemibiotrophic fungal pathogen *M. oryzae* because (i) *M. oryzae* proliferates biotrophically during the initial 72 hours of the infection (Wilson and Talbot, 2009) and (ii) rice blast is the most damaging fungal disease for rice production worldwide (Dean et al., 2012). We microscopically monitored and rated early invasive, biotrophic blast growth on leaf sheaths of 1-month-old plants. Invasive hypha development was delayed in the four transgenic lines 8, 11, 16, and 19 compared to sib lines without *Lr34res* (Figure 1a; Figure S2). Most infection sites showed low levels of invasive growth (levels 1 and 2) in the transgenic lines after 28 hours and only 15% - 29% of the penetration sites

developed invasive hyphae of levels 3 and 4 (high levels of invasive growth). In contrast, 75% of the penetration sites showed high levels of invasive growth (levels 3 and 4) in the sib lines after 28 hours. After 40 hours, more than 40% of the infection sites spread to multiple cells (level 4) in the sib lines, compared to less than 10% after 28 hours (Figure S3). The delay in pathogen development by *Lr34res* was still apparent 40 hours post infection and only 12% of the penetration sites spread to more than one cell in the transgenic line 19. This slow-down in disease development in *Lr34res*-containing rice is very similar to the partial resistance characteristic for *Lr34res* in wheat and barley. The multi-copy line 5 showed silencing of *Lr34res*-based disease resistance. While T2 plants showed a reduction in disease progression, there was no difference between transgenic and sister plants at the T5 generation (Figure S4). To test whether the delay in early invasive growth was sufficient to also reduce macroscopic rice blast symptoms, we spray-inoculated rice plants and evaluated necrotic lesions seven days after infection. The four transgenic lines 8, 11, 16 and 19 developed less and smaller lesions compared to sib lines when infected with *M. oryzae* isolate FR13 (Figure 1b; Figure S5). Furthermore, lines 16 and 19 were tested with three additional rice blast isolates originating from Korea and China. Similar to the results obtained with FR13, transgenic lines showed reduced lesion formation compared to the sib lines for all three isolates (Figure 1c). These results show that *Lr34res* in rice confers partial resistance against multiple rice blast isolates.

In wheat and barley, *Lr34res* is associated with a senescence-like process called leaf tip necrosis (LTN, Krattinger et al. 2009). Transgenic rice plants also developed the typical LTN (Figure 2a; Figure S6). In contrast to barley where LTN was very strong and ultimately affected entire leaves (Risk et al., 2013), LTN in rice was restricted to the distal part of the leaf. The rice phenotype resembles the LTN found in adult wheat plants rather than the uncontrolled necrosis of barley. Lines 11, 16 and 19 developed LTN from early seedling stage on with the strongest LTN observed in line 19. LTN development in this particular line started at the two-leaf stage when the plants were approximately ten days old. LTN first developed on the older lower leaves and subsequently appeared on younger upper leaves as the plants matured. The early development of LTN had a severe negative impact on the formation of axillary shoots, overall plant vigor and spikelet production (Figure 2b, Table S1). The most severe phenotype was observed in line 19. While plants of sib lines had between 10 and 15 fertile tillers on average (Table S1), transgenic line 19

developed only 5 tillers on average. Line 8 on the other hand did not develop LTN at seedling stage but only in adult plants after about one month. Similar to the other lines, LTN first emerged on lower leaves and subsequently on upper leaves. In contrast to the lines with early LTN development, axillary shoot formation in line 8 was not or only marginally compromised. Plants of transgenic line 8 had 12 tillers on average whereas plants of the respective sib line had 14.8 tillers per plant on average (T-Test P=0.115, n=6, Table S1). The number of spikelets per panicle did not differ between line 8 (43 spikelets / panicle) and the sib line (46 spikelets / panicle, T-Test P=0.25, n=6, Table S1) but was significantly reduced in lines 16 and 19. Both lines 19 and 8 carry single T-DNA insertions. The difference in strength of LTN can therefore not be attributed to variation in copy number (Figure S1). RT-qPCR analysis revealed that line 8 showed *Lr34res* expression levels that were 4 to 10 times lower than in the other three lines at seedling stage (Figure 3). *Lr34res* expression increased in adult plants of line 8 about five times and reached levels comparable to those of the other lines. *Lr34res* expression levels between the other three lines 11, 16 and 19 did not significantly differ at seedling and adult plant stages. Hence, the late development of LTN in line 8 can most likely be explained by the lower *Lr34res* expression levels at early growth stage. Importantly, the level of LTN at seedling stage did not correlate with level of disease resistance. Although line 8 showed the weakest LTN development, it showed resistance levels comparable to the other lines or even stronger (Figure 1a).

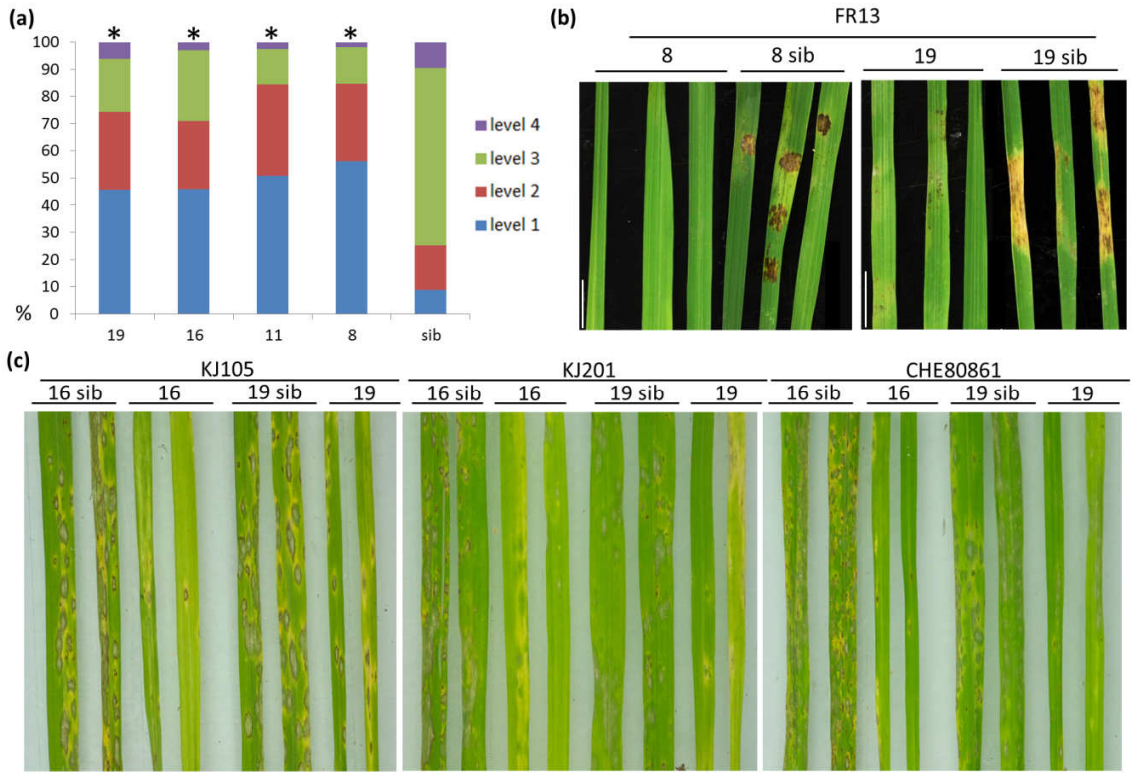

Figure 1. *Lr34res* confers partial resistance against *M. oryzae* in transgenic rice. (a) Classification of biotrophic *M. oryzae* development according to Saitoh et al. (2012) on rice leaf sheaths 28 hours after infection. Levels 1 to 4 represent different lengths of invasive hyphae with level 1 being the shortest (see Experimental procedures for detail). The y-axis shows the percentages of appressorial penetration sites that belong to the different infection levels. At least three biological replicates were averaged for each line and ~50 appressorial penetration sites were evaluated for each replicate. * indicate significant differences compared to sib lines (sib = pool of sibs from the four transgenic lines; Mann–Whitney U-test, P<0.01). (b), (c) Macroscopic development of different *M. oryzae* isolates on different transgenic lines and sib lines 7 days post infection. Infection experiments in (b) were done by drop-inoculation at the University of Zurich and (c) by spray-inoculation at the Zhejiang Academy of Agricultural Sciences, China. Isolate FR13 was collected in France, KJ105 and KJ201 in Korea and CHE80861 in China. Scale bar in (b) = 10mm.

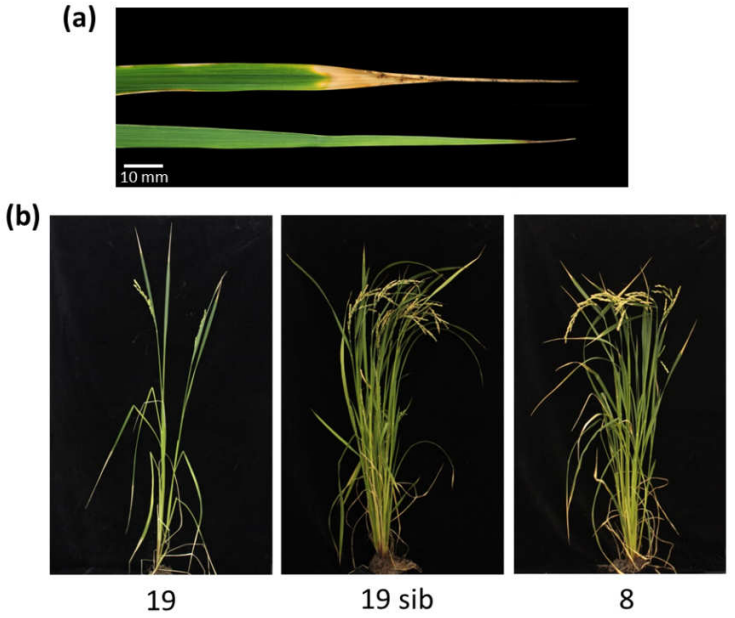

Figure 2. *Lr34res* results in a typical, senescence-like leaf tip necrosis (LTN) in transgenic rice.
(a) Representative example of leaf tip necrosis on flag leaves of transgenic line 19 (upper leaf) compared to its sib line (lower leaf). Scale bare = 10 mm. (b) Early LTN development results in suppressed axillary shoot formation in line 19 (left). Line 8 only developed LTN at adult plant stage and axillary shoot formation in this line was not affected (right).

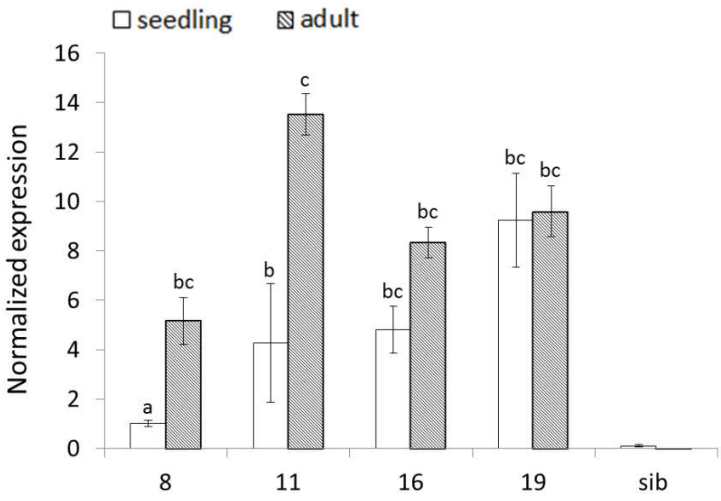

Figure 3. Normalized *Lr34res* expression in transgenic lines. Letters indicate lines with equivalent expression levels (Tukey's honest significance test, P>0.05). Data are normalized to the reference gene *UBC1* (Jain et al., 2006) from three biological replicates and error bars represent standard error.

2.2 – Site-directed mutagenesis of the orthologous rice *Lr34* gene

Rice cultivar Nipponbare contains an expressed orthologous *Lr34* gene – *OsABCG50* – with an intact open reading frame (Krattinger et al. 2011). The predicted OsABCG50 protein shares 86% amino acid identity with LR34res. Because an *Lr34*-like, race non-specific, multi-pathogen disease resistance with LTN has not been reported in rice, we hypothesize that OsABCG50 has a function different from disease resistance. This hypothesis is supported by the fact that OsABCG50 carries the susceptible haplotype for the two critical amino acid residues that distinguish LR34res from LR34sus in wheat (Krattinger et al., 2013; Figure S7a). At the first position located in exon 11, *OsABCG50* has the nucleotide triplet 'TTA' encoding for a leucine residue (L550). LR34sus carries a phenylalanine at the orthologous position (F546). These two residues are predicted to be located within a transmembrane helix and only LR34res has a deletion of a hydrophobic amino acid at this position (Krattinger et al. 2013). At the second critical position, both LR34sus and OsABCG50 carry a tyrosine residue encoded by 'TAT' that is converted to a histidine in LR34res ('CAT'). According to Genevestigator (Zimmermann et al., 2008) *OsABCG50* shows medium expression levels in all parts of the rice plant that are affected by rice blast, namely leaves, sheaths and panicles. We tested whether site-directed modification of these two residues in OsABCG50 might alter its function and result in broad-spectrum *M. oryzae* resistance. For this, we constructed the resistant OsABCG50 haplotype by deleting the leucine residue (ΔL550) and by converting the critical tyrosine to histidine (Y638H). A plasmid containing the altered allele - named *OsABCG50res* - was stably transformed into the *japonica* rice cultivar Nipponbare. *Lr34res* in wheat functions in the presence of *Lr34sus* and we therefore expected that the presence of the endogenous *OsABCG50* copy would not interfere with a potential involvement of *OsABCG50res* in *M. oryzae* resistance. We identified two transgenic events (4.2 and 9.1; Figure S7b) that expressed *OsABCG50res*. Segregating T2 families of these two events were infected with rice blast isolate FR13 and early disease progression was monitored microscopically. There was no difference in early *M. oryzae* invasion between lines with *OsABCG50res* and segregating sib lines (Figure S7c). Furthermore, plants expressing *OsABCG50res* also did not develop LTN. These experiments indicate that targeted modification on *OsABCG50* based on the corresponding amino acid changes in *Lr34res* does not result in rice blast resistance.

(3) Discussion

In this study we showed that the wheat durable, multi-pathogen resistance gene *Lr34* confers resistance against multiple *M. oryzae* isolates in rice. Rice, together with wheat and maize, is one of the three most widely grown crops in the world, providing a daily food source for more than half of the global population. Rice blast is the most devastating and most economically damaging rice disease (Wilson and Talbot 2009). It is a rapidly evolving pathogen and occurrence of new *M. oryzae* races frequently resulted in breakdown of rice blast resistance in the past (Dean et al., 2012; Huang et al., 2014; Wilson and Talbot, 2009). More than 90 rice blast resistance genes have been identified in cultivated and wild rice germplasm until today and 22 of them have been cloned. Most of the cloned blast resistance genes confer race-specific resistance and code for NBS-LRR immune receptors (Fukuoka et al., 2014; Wang et al., 2014). There are only very few examples of rice blast resistance genes that are active against a broad range of *M. oryzae* isolates. For example, rice plants containing the NBS-LRR gene *Pi9* are resistant against more than 100 rice blast isolates (Qu et al., 2006). Similarly, the atypical NBS-LRR gene *Pb1* confers race non-specific and durable blast resistance at adult plant stage. It is not yet understood why the pathogen cannot adapt to the *Pb1* resistance gene (Hayashi et al., 2010; Inoue et al., 2013). A third example is *Pi21* that encodes for a protein with a putative heavy metal-binding domain and a protein-protein interaction motif. This gene represents a susceptibility factor and loss-of-function of *Pi21* results in durable, race non-specific and recessive rice blast resistance (Fukuoka et al., 2009).

The origin of *Lr34* in modern wheat breeding goes back to crosses made in Italy by the wheat breeder Nazareno Strampelli in the early 20th century (Kolmer et al., 2008). *Lr34* has been extensively used in breeding since the Green Revolution and it continues to provide race non-specific and partial field resistance against the three wheat rust diseases and powdery mildew. *Lr34* therefore fulfills all the criteria of a durable disease resistance gene in wheat because it remained effective during its prolonged and widespread use in environments favorable to the disease (Johnson, 1984). We therefore consider it likely that the partial resistance described in this study might also be durable in rice. In wheat, *Lr34* is often combined with other partial adult plant resistance genes and this strategy resulted in wheat cultivars that show near-immune levels of broad-spectrum disease resistance (Ellis et al., 2014). Pyramiding of partial resistance genes has also been successfully used to enhance blast resistance in rice (Fukuoka et al., 2015). Rice blast

infections can occur during all stages of rice development on leaves, stems and panicles (Wilson and Talbot, 2009). Our results showed that *Lr34* confers effective rice blast resistance on sheaths and leaves. *Lr34* might therefore be a valuable source to improve broad-spectrum rice blast resistance in rice cultivars in the future. The combination of *Lr34* with other broad-spectrum blast resistance genes like *pi21* or *Pb1* would be the most sustainable strategy to achieve long lasting and effective blast resistance.

Despite the limited number of independent transgenic events generated during this study, we recovered a line that showed late leaf tip necrosis and high levels of disease resistance but no obvious negative impact on tiller formation and spikelet development. This is in contrast to barley, where all transgenic lines showed a dramatic reduction in growth and seed production (Risk et al., 2013). Our data indicated that high *Lr34res* expression levels at seedling stage correlated with early LTN development and reduced plant vigor. Low seedling expression of *Lr34res* in line 8 on the other hand resulted in late LTN development and normal plant growth. The detrimental effects of early *Lr34res* expression might also explain why *Lr34res* in wheat evolved as an adult plant resistance gene that is not effective at seedling stage.

Wheat and rice shared their last common ancestor 40-54 million years ago (The International Brachypodium Initiative, 2010). The rice *Lr34* ortholog *OsABCG50* is most likely not involved in rice blast resistance and has the susceptible *Lr34* haplotype. It is therefore remarkable that all components required for the function of *Lr34res* are still present in rice. This suggests that the substrate transported by *Lr34* must be conserved in wheat, barley and rice. An obvious experiment was the artificial reproduction of the evolutionary events that gave rise to *Lr34res* in wheat by site-directed mutagenesis of *OsABCG50*. A successful outcome might have opened the possibility to increase durable blast resistance in rice non-transgenically through targeted genome editing of *OsABCG50* by TALEN or CRISPR/Cas. Our results however suggested that besides the two critical residues, additional sequence motifs of *Lr34res* might be required for disease resistance in rice. The replacement of larger *OsABCG50* segments spanning the two critical nucleotide triplets with the corresponding wheat sequence might still result in rice blast resistance since the transformation of the entire wheat *Lr34res* gene was functional. A future approach might therefore consist in the generation and testing of multiple rice *OsABCG50* -wheat *Lr34res* chimeric constructs to identify the smallest functional segmental replacement.

So far, *Lr34* was associated with resistance to obligate biotrophic pathogens of wheat and barley. In this study we could show that *Lr34* also functions against a hemibiotrophic pathogen that has a different lifestyle from rusts and mildews. Obligate biotrophic pathogens remain in close association with their host cells throughout the entire infection cycle. *M. oryzae* represents a special case of hemibiotrophic lifestyle that lacks a distinct switch from biotrophy to necrotrophy. Instead, the growing front of the fungus remains biotrophic as it spreads from cell to cell but invaded cells lose viability by the time the fungus moves to the next cell (Kankanala et al. 2007). The inhibition of this biotrophic rice blast growing front by *Lr34res* is obviously sufficient to reduce macroscopic disease symptoms on rice leaves.

In this study, we show a proof of principle that *Lr34res* might serve as an effective source to increase broad-spectrum and durable rice blast resistance. Future field tests and infection experiments with additional relevant rice diseases will be necessary to assess the full benefit of *Lr34res* for rice breeding.

(4) Experimental procedures

4.1 – Plasmid construction

The cloning of the genomic sequence of the resistant *Lr34res* allele with its native promoter and terminator into the binary vectors p6U and pWBVec8 was described in Risk et al. (2013) and resulted in plasmids *p6U:gLr34res* and *pWBVec8:gLr34res*, respectively. The genomic *OsABCG50* sequence including 2,879 bp native promoter and 2,025 bp terminator sequence was released from Nipponbare BAC clone OsJNBa0035B12 by *Eag*I digestion. The 16,521 bp *Eag*I fragment was sub-cloned into vector pWGEM-NZf(+) (Promega). The entire cassette was then transferred into vector pWBVec8 (Wang et al., 1998) through *Not*I. For the site-directed mutagenesis, a 2.6 kb fragment of *OsABCG50* was released from *pWGEM-NZf(+):OsABCG50* with a *Sac*II-*EcoR*I double digest and cloned into pGEM®-T Easy vector (Promega) digested with the same enzymes. To introduce the two mutations, two rounds of site-directed mutagenesis were performed using the Quick Change XL Site-Directed Mutagenesis kit (Agilent Technologies) with primers 5’ GGA GCA TTG TTT TTT TCC ATC ATG ATC ATG CTA AAT GGC ACA -3’ (ΔL550) and 5’ CAT CAA TCA GTC ACT TCG CTG CAT CGA TTT ATT GCT TCA TAC TT 3’ (Y638H). The altered *Sac*II-*EcoR*I fragment was then

re-introduced into the digested *pWBVec8:OsABCG50* fragment to result *in pWBVec8:OsABCG50res*.

4.2 – Agrobacterium-mediated transformation and selection of transgenic events

The binary plasmids *p6U:gLr34res* (events 5, 8, 11 and 16) and *pWBVec8:OsABCG50res* were transformed into the *Agrobacterium tumefaciens* strain EHA105 by electroporation according to Sambrook and Russel (2001). Fifty primary calli of Nipponbare were transformed as described in Sallaud et al. (2003). Plasmid *pWBVec8:gLr34res* (event 19) was cloned into Agrobacterium strain AGL1. Transformation with this plasmid was done according to Upadhyaya et al. (2000). Primary transgenic T0 plants transformed with *Lr34res* were evaluated for T-DNA integration using the *Lr34res*-specific PCR marker *cssfr1* (Lagudah et al., 2009). A primer pair that specifically distinguished *OsABCG50res* from the endogenous *OsABCG50* gene was used to check for insertion and expression of the *OsABCG50res* T-DNA (OsABCG50res_f: 5'- GAG CAT TGT TTT TTT CCA TCA TG-3' and OsABCG50res_r: 5'- GCA GCG AAG TGA CTG ATT GAT G-3'). PCR positive T0 plants were evaluated for expression of the transgenes using *Lr34res*-specific cDNA primers (Risk et al., 2013) or OsABCG50res_f – OsABCG50res_r. To determine copy number, 7.5 µg of genomic DNA of T0 and T1 plants was digested with *Xba*I (pWBVec8) and *EcoR*I or *Apo*I (p6U). Southern Blots were done as described in Risk et al. (2013). Homozygous T2 lines for *Lr34res*-containing events were PCR-selected using *cssfr1* on ~20 individual T2 plants.

4.3 – Fungal strain cultivation

M. oryzae isolate FR13 was grown on oatmeal agar (30 g/l oatmeal, 5 g/l sucrose and 16 g/l agar) at room temperature, with 12 h light during 5 days. In order to enhance sporulation, the fungus was transferred to white light / blue light (Philips TL-D 15W BLB) for 4 additional days. Rice blast conidia were then harvested from plates by rinsing with sterile distilled water and raking with a spatula. Spores were filtered through two layers of gauze and suspended to a final density of 1 x 10^5 conidia/ml.

4.4 – Leaf sheath infection assay

Rice plants were grown in a growth cabinet at 28°C / 24°C day / night and 75% humidity. Early development of *M. oryzae* was monitored microscopically according to Saitoh et al. (2012). In brief, the inoculation of leaf sheath was performed with a syringe on a 3 cm leaf sheath fragment of 5-week-old plants using a concentration of 1 x 10^5 conidia/ml of isolate FR13. Infected fragments were then incubated for 28 hours or 40 hours in a humid petri dish at room temperature in the dark. To investigate the level of infection, invasive hyphae were stained with lactophenol-trypan blue (30 ml ethanol, 10 ml glycerol, 10 ml lactic acid, 10 mg trypan blue and 10 ml distilled water). Whole leaf sheath segments were boiled for 1 minute in the staining solution and decolorized in chloral hydrate (2.5 g of chloral hydrate in 1 ml ddH_2O) for at least 30 minutes. 50 appressorial sites were evaluated per leaf sheath using a Zeiss Axio Imager Z1 microscope. The different levels of invasive growth were classified according to Saitoh et al., (2012): Level 1, invasive hypha length is shorter than 10 µm with no branch; Level 2, invasive hypha length is 10–20 µm with 0–2 branches; Level 3, invasive hypha length is longer than 20 µm and/or with more than 2 branches within one cell; Level 4, invasive hypha is spread into more than one cell.

4.5 – Rice blast spray infection

3-week-old seedling plants were spray-inoculated with spore solutions of *M. oryzae* isolates FR13 (France), KJ105 (Korea), KJ201 (Korea) and CHE80861 (China). A conidial suspension of 1x 10^5 spores/mL in gelatin (0.1% w/v) / tween20 (0.01% w/v) was sprayed on seedling plants. In addition, 3 µl droplets of FR13 spore solution were added directly on the leaves. Plants were covered with a plastic tent in order to maximize humidity and they were kept in a greenhouse with temperature at 28°C and a 12 hour photoperiod. Disease symptoms were evaluated 7 days after infection.

4.6 – Quantitative expression analysis

Expression analysis was done using RT-qPCR. Total RNA was extracted from rice leaves using the SV Total RNA Isolation System (Promega). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and RNA integrity was assessed on a 1.2% SB agarose gel. cDNA was synthesized from 1 µg of RNA using the i-Script™ cDNA Synthesis kit (BioRad).

Expression of *Lr34res* was measure using the *Lr34res* qPCR probe designed by Risk et al. (2012). RT-qPCR was done in a 10 µl reaction including 5 µ KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems), 4 uL of 1:20 diluted cDNA template and 500 nM final concentration of forward and reverse primers. Samples were run on a CFX96 Touch™ Real-Time PCR Detection System (BioRad). The ubiquitin-conjugating enzyme E2 (*UBC*; LOC_Os02g42314; UBC_f 5'-GAA TGT GCA TTT CAA GAC AGG-3', UBC_r 5'-GCA ATT ATG GCT CTA CAA ACG G-3') was used as reference gene (Jain et al., 2006). Primers for *Lr34res* and *UBC* were designed on exon-intron junctions to avoid amplification of genomic DNA. Thermocycling conditions for both probes were 95°C for 1 minute, followed by a two-step PCR of 40 cycles of 95°C for 3 sec and 65°C for 20 sec. Primer efficiencies were measured through serial dilutions and no reverse transcriptase (NRT) controls were included in each PCR run. RT-qPCR was performed on at least three biological replicates with technical triplicates. Data were analyzed using the CFX Manager 3.1 software (BioRad). Statistical analysis was done using the REST software (Pfaffl et al., 2002) and the JMP® statistical package (SAS Institute).

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(7) Supplementary Figures

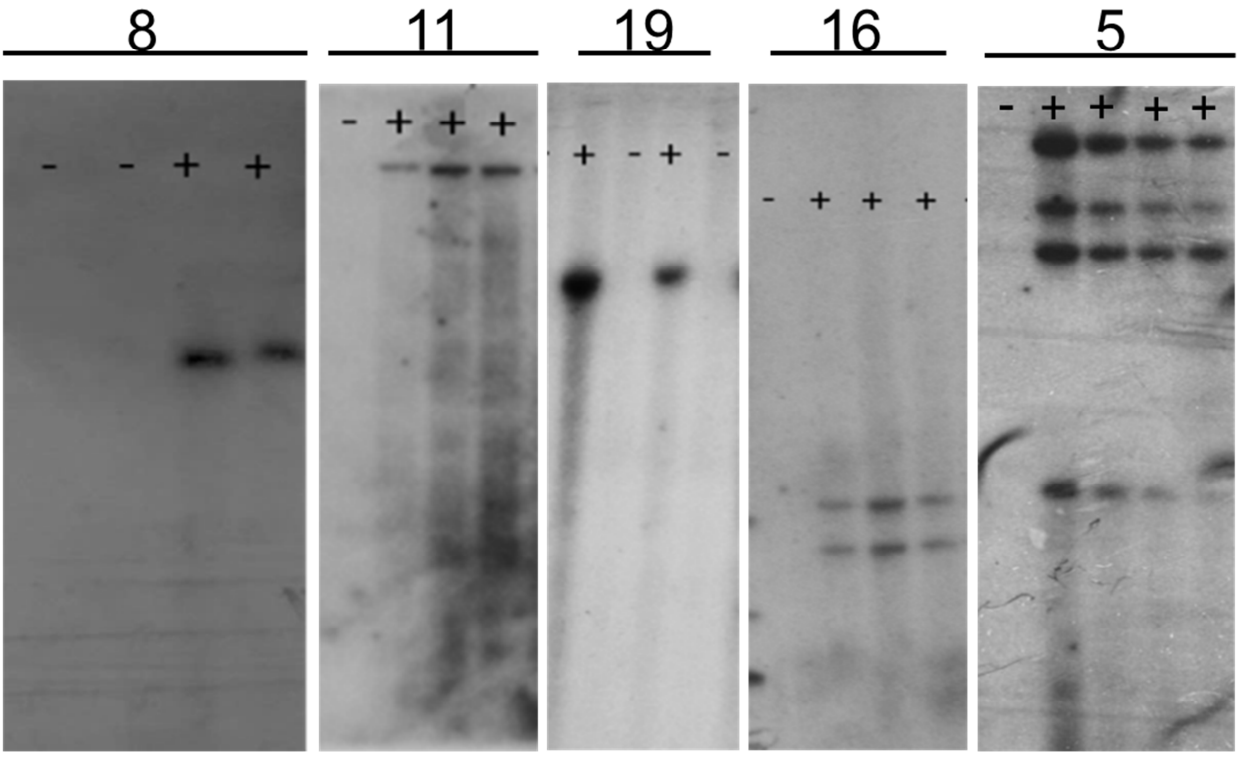

Figure S1 Southern Blot showing T-DNA copy number in the five independent transgenic *Lr34res* rice lines. + indicates plants with *Lr34res* and –sib lines without *Lr34res*.

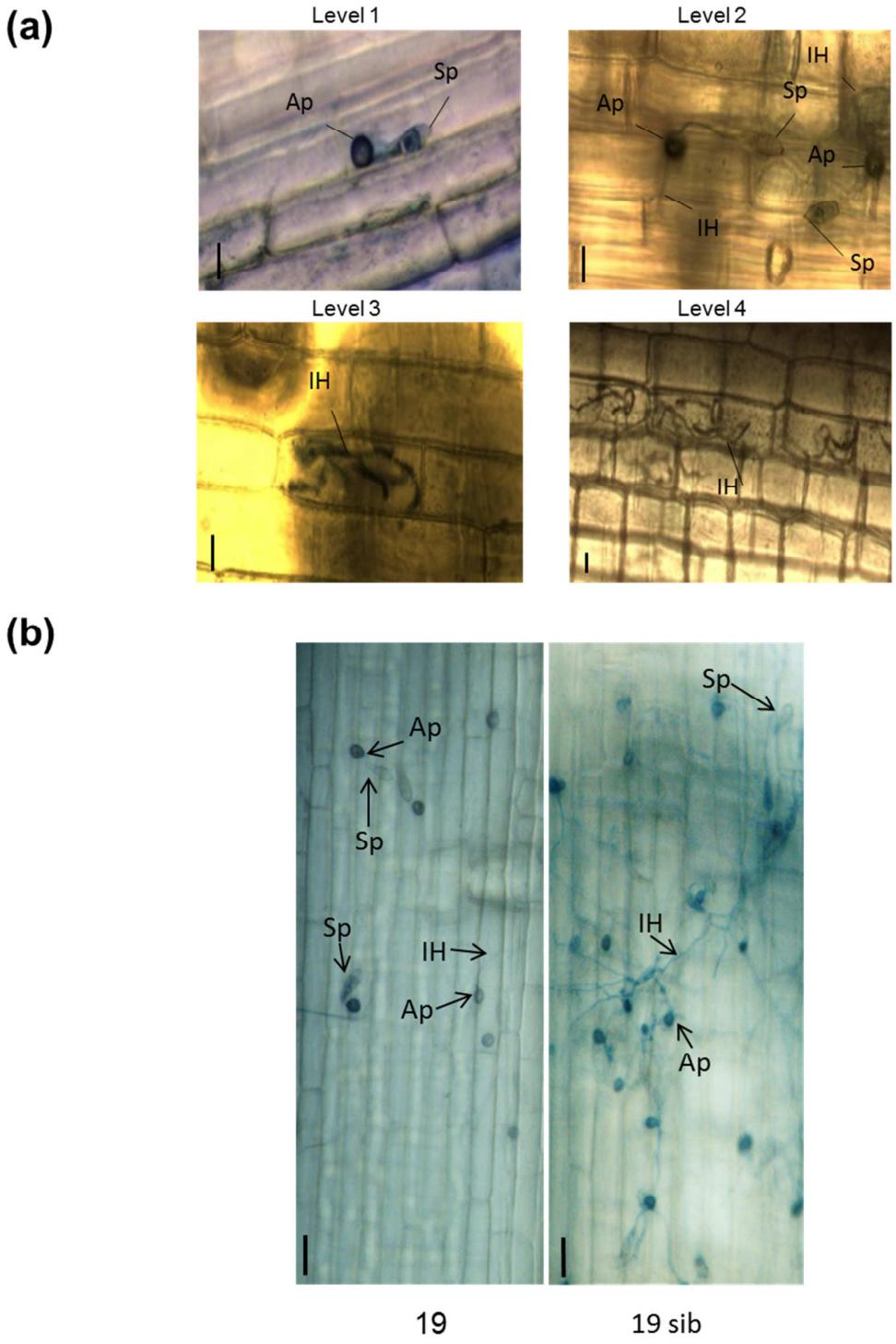

Figure S2 (a) Microscopic images of the four levels of early *M. oryzae* invasive growth. Level 1, invasive hypha length is shorter than 10 µm with no branch; Level 2, invasive hypha length is 10–20 µm with 0–2 branches; Level 3, invasive hypha length is longer than 20 µm and/or with more than two branches within one cell; Level 4, invasive hypha is spread into more than one cell. (b) Representative images of leaf sheath surface of lines 19 and 19 sib 28 hours after *M. oryzae* infection. Most appressorial infection sites in line 19 are levels 1 and 2, whereas 19 sib shows many infection sites of levels 3 and 4.
Sp = spore; Ap = appressorium; IH = invasive hypha; scale bar = 15 µm for (a) and (b)

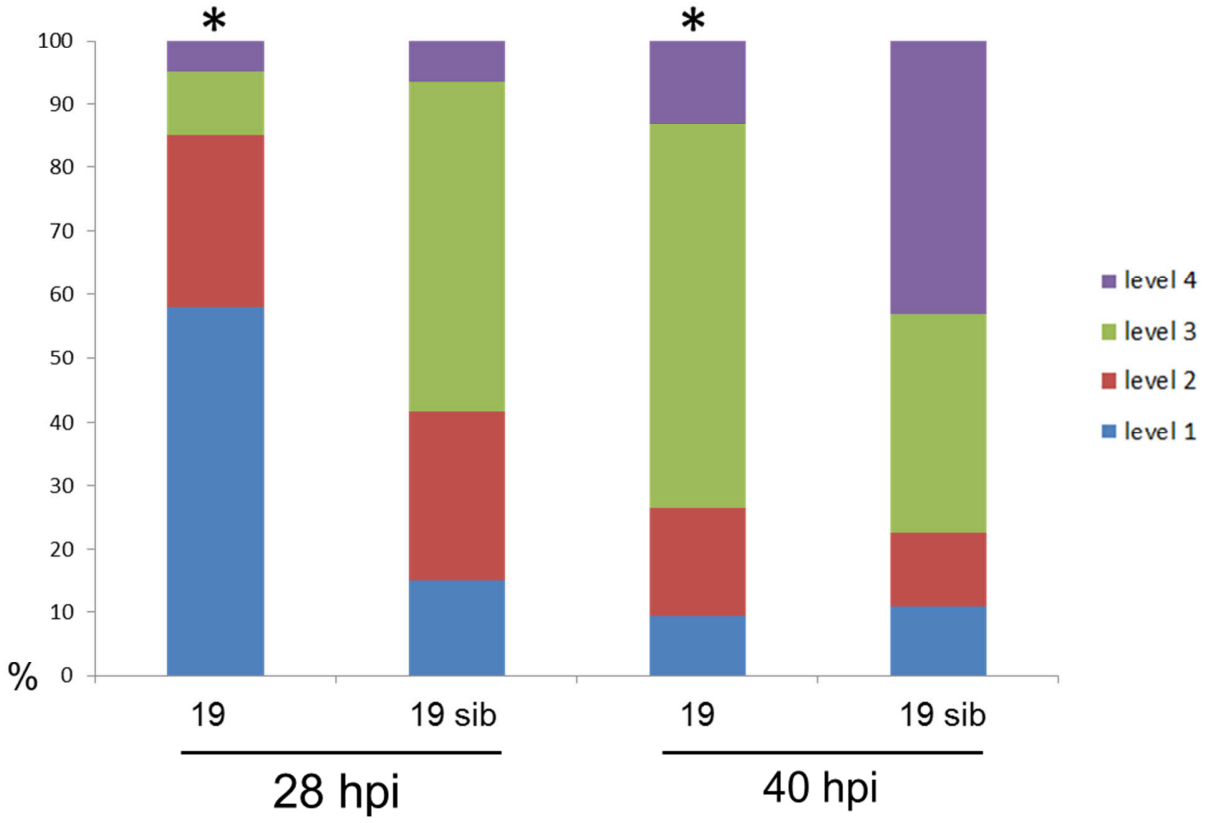

Figure S3 Comparison of early invasive *M. oryaze* growth in transgenic line 19 and corresponding sib line (sib) 28 hours (left) and 40 hours (right) post infection (hpi).
Levels 1 to 4 represent different lengths of *M. oryzae* invasive hyphae with level 1 being the shortest (see Experimental procedures for detail). Three biological replicates were averaged and ~50 appressorial penetration sites were evaluated for each replicate. * indicates a significant difference compared to the respective sib line (Mann–Whitney U-test, $P<0.01$).

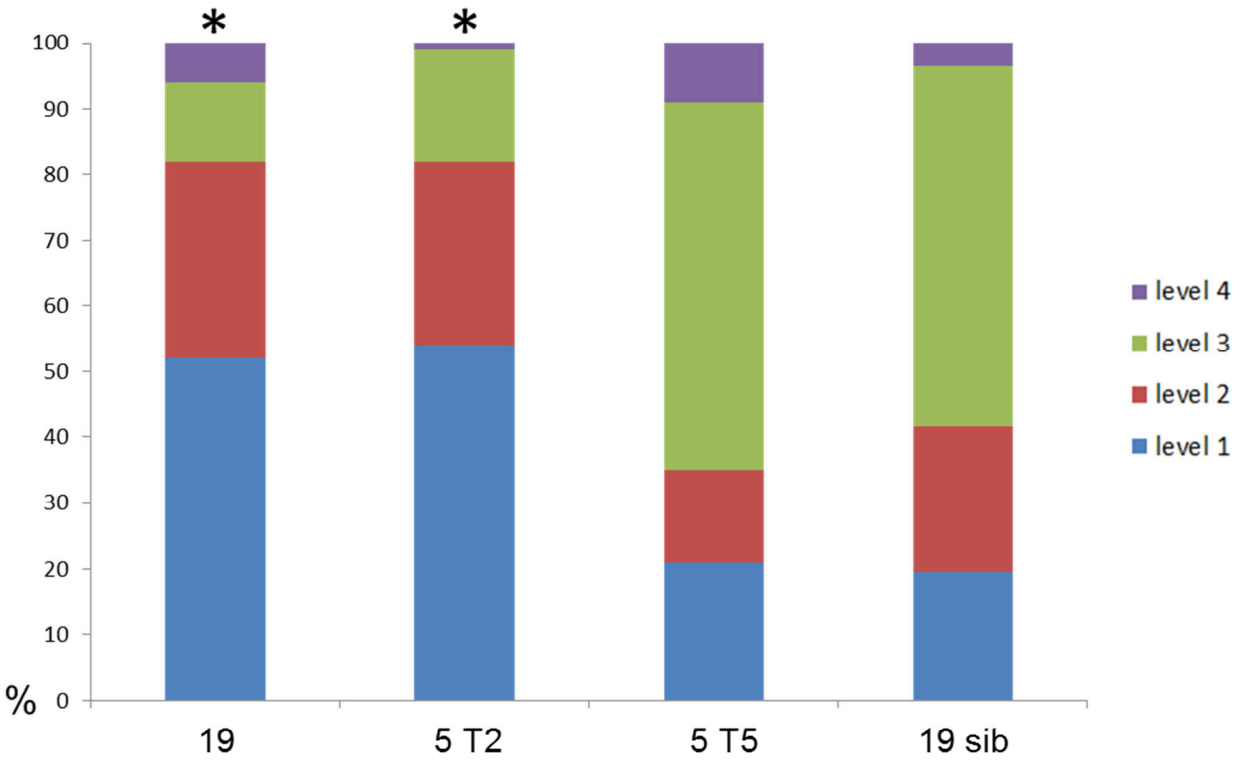

Figure S4 Silencing of disease resistance phenotype in the multi-copy line 5. Microscopic *M. oryzae* development in T2 (resistant) and T5 (silenced) generation of line 5 is compared to line 19 and its sib line 19 sib 28 hours after infection. Levels 1 to 4 represent different lengths of *M. oryzae* invasive hyphae with level 1 being the shortest (see Experimental procedures for detail). Three biological replicates were averaged and ~50 appressorial penetration sites were evaluated for each replicate. * indicates a significant difference compared to 19 sib (Mann–Whitney U-test, $P<0.01$).

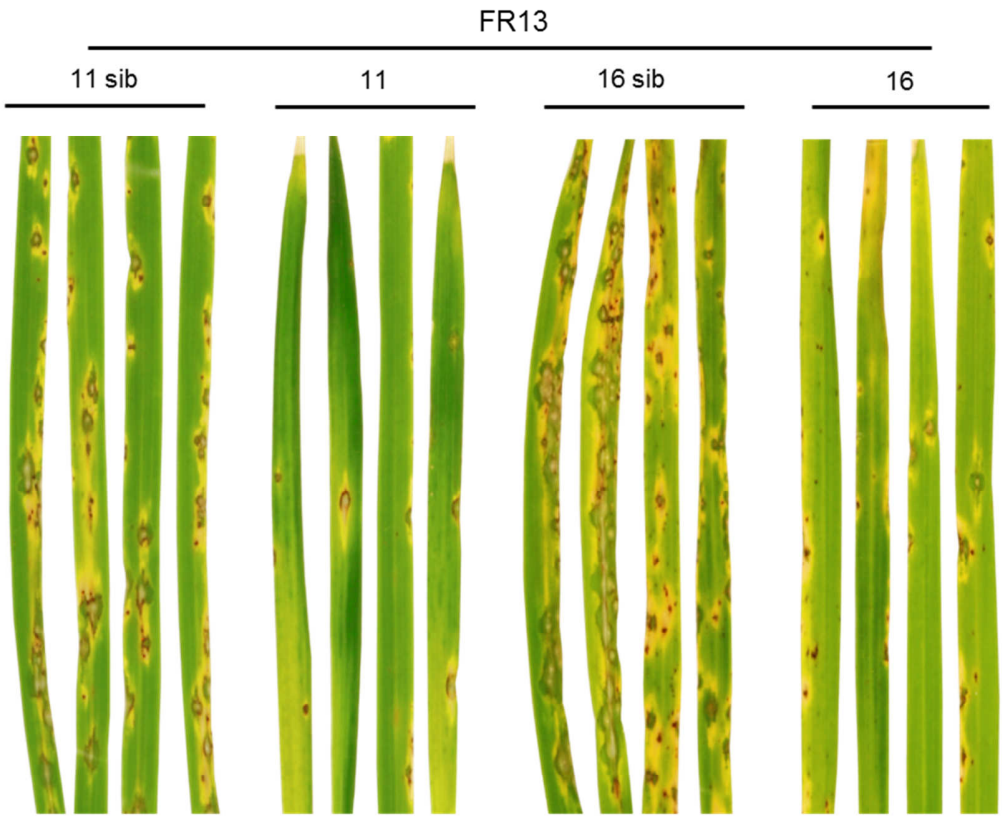

Figure S5 Representative examples of leaves of transgenic lines 11 and 16 and corresponding sib lines (sib) infected with *M. oryzae* isolate FR13, 7 days post infection.

Figure S6 Leaf tip necrosis on flag leaves of the four *Lr34res* lines 8, 11, 16 and 19. Scale bar = 10mm.

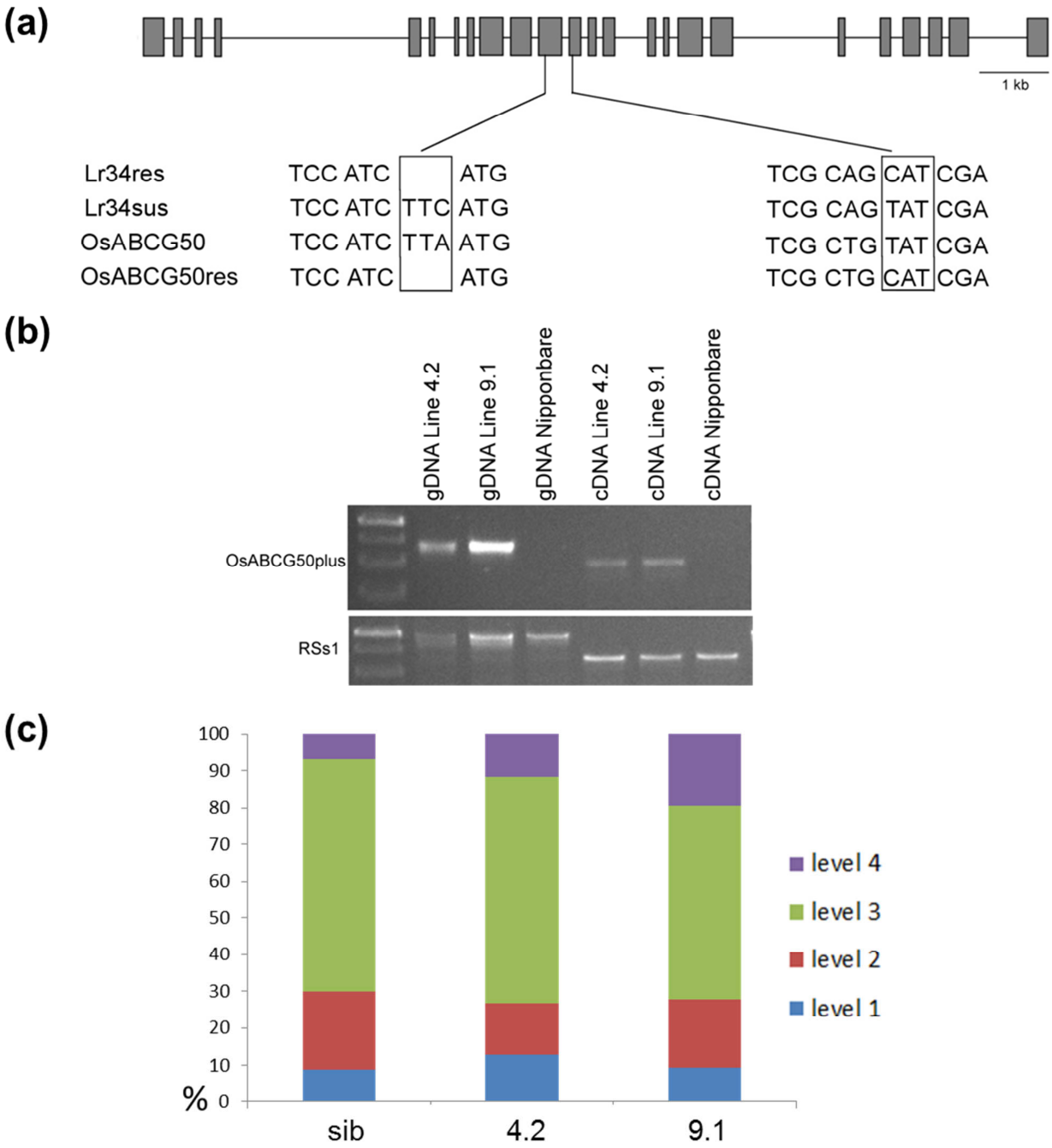

Figure S7 Site-directed modification of the rice *Lr34*-ortholog *OsABCG50* does not result in increased *M. oryzae* resistance. (a) Schematic representation of the *Lr34* exon-intron structure with exons depicted as grey boxes and introns as lines. The nucleotide sequences at the two critical positions in exons 11 and 12 are shown for *Lr34res*, *Lr34sus*, *OsABCG50* and *OsABCG50res*. The schematic exon-intron structures of *OsABCG50* and *Lr34* are identical. (b) Presence and expression of *OsABCG50res* in transgenic lines 4.2 and 9.1. Expected fragment sizes of the *OsABCG50res* specific PCR product are 377 bp on genomic DNA (gDNA) and 285 bp on cDNA. The rice sucrose synthase-1 gene (RSs1) was used as control. The visible bands of the GeneRuler™ 1kb Plus DNA Ladder (Life Technologies) correspond to 500 bp, 400 bp, 300 bp and 200 bp from the top, respectively (c) Microscopic evaluation of early *M. oryzae* development on *OsABCG50res* lines 4.2 and 9.1. Levels 1 to 4 represent different lengths of *M. oryzae* invasive hyphae with level 1 being the shortest (see Experimental procedures for detail). Three biological replicates were averaged and ~50 appressorial penetration sites were evaluated for each replicate. * indicates a significant difference to the sib lines (sib = pooled plants of 4.2 sib and 9.1 sib; Mann–Whitney U-test, $P<0.01$).

Table S1 Number of tillers and number of spikelets per panicle for three transgenic lines compared to their respective sib lines. 8/8 sib were gown in different conditions than 16/16 sib and 19/19 sib. Asterisks indicate significant differences between transgenic line and respective sib (Student's T-Test, P<0.05).

Line	Number of Plants	Number of Tillers	Number of Spikelets per Panicle
8	6	12 ± 1.4	43.3 ± 1.6
8 sib	6	14.8 ± 0.9	46.1 ± 1.8
16	6	8 ± 1.6	22.1 ± 1*
16 sib	6	10 ± 1.4	29.2 ± 1.8
19	4	5.25 ± 0.5*	20.2 ± 1.6*
19 sib	5	10.2 ± 1.2	29 ± 1.3

Chapter C

The durable wheat disease resistance gene *Lr34* confers common rust and northern corn leaf blight resistance in maize

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Summary

Maize (corn) is one of the most widely grown cereal crops globally. Fungal diseases of maize cause significant economic damage by reducing maize yields and by increasing input costs for disease management. The most sustainable control of maize diseases is through the release and planting of maize cultivars with durable disease resistance. The wheat gene *Lr34* provides durable and partial field resistance against multiple fungal diseases of wheat, including three wheat rust pathogens and wheat powdery mildew. Because of its unique qualities, *Lr34* became a cornerstone in many wheat disease resistance programs. The *Lr34* - resistance is encoded by a rare variant of an ATP-binding cassette (ABC) transporter that evolved after wheat domestication. An *Lr34* - like disease resistance phenotype has not been reported in other cereal species, including maize. Here, we transformed the *Lr34* resistance gene into the maize hybrid Hi-II. *Lr34* - expressing maize plants showed increased resistance against the biotrophic fungal disease common rust and the hemi-biotrophic disease northern corn leaf blight. Furthermore, the *Lr34* - expressing maize plants developed a late leaf tip necrosis phenotype, without negative impact on plant growth. With this and previous reports it could be shown that *Lr34* is effective against various biotrophic and hemi-biotrophic diseases that collectively paracitise all major cereal crop species.

(1) Introduction

The world population is estimated to increase from currently 7.4 billion people to 9.6 - 12.3 billion people by the end of this century (Gerland *et al.* 2014). In order to meet the rising demands for food, feed and biofuels, a doubling in crop production will be necessary (Alexandratos and Bruinsma 2012). Maize (corn) is one of the most widely grown crops and maize production passed the mark of 1,000 million metric tons in 2013 (FAO, 2016; de Lange *et al*. 2014). Maize is used for human consumption, as a major source of animal feed (Chaudhary et *al.* 2014) and for the production of a number of industrial products such as starch, oil, ethanol and malt. Maize is grown in more than 160 countries over a wide range of agro-climatic zones.

Fungal diseases are a major threat to maize production, resulting in reduced yields and high input costs for disease control. Global maize yield losses caused by diseases were estimated at about 9% on average in 2001-2003 (Oerke 2006). Sixty-two maize-infecting diseases have been described and among them sixteen have been identified as major constraints for maize production (Chaudhary *et al*. 2014). The hemi-biotrophic fungal disease northern corn leaf blight (NCLB), also known as Turcicum leaf blight (*Exserohilum turcicum*) and the maize common rust disease caused by the biotrophic fungus *Puccinia sorghi,* are among these most important foliar diseases (Zwonitzer *et al*. 2010; Brown *et al*. 2001). Biotrophic and hemi-biotrophic fungi have different lifestyles. Obligate biotrophs such as rusts and powdery mildews exclusively grow and reproduce on living plant tissue (Eckardt *et al*. 2006; Bettgenhaeuser *et al*. 2014). In contrast, hemi-biotrophic fungi feed on dead leaf tissue after an initial biotrophic phase that can last from a few hours to several days.

The release of cereal cultivars with high levels of durable field resistance is one of the most effective and sustainable strategies to control fungal diseases (Mc Donald *et al*. 2002). Hundreds of fungal disease resistance genes have been described in various cereal species (Singla and Krattinger 2016; Wisser *et al*. 2006). Fungal disease resistance genes can be broadly classified based on their specificity and durability. Race-specific resistance (*R*) genes are only effective against some isolates of a pathogen whereas race non-specific resistance is effective against all pathogen isolates. The former is often controlled by a single major *R* gene encoding for plasma membrane-localized or intracellular immune receptors and the latter is often the result of the additive action of several quantitative resistance genes with minor phenotypic effects (Krattinger

and Keller 2016). The deployment of single *R* genes in one cultivar often results in breakdown of disease resistance because of rapid pathogen adaptation. The combination of several race-specific *R* genes in one cultivar, the use of multiline cultivars with different resistance gene combinations and the use of more durable resistance sources are effective strategies to increase the durability of disease resistance in the field (Brunner *et al.* 2012).

In hexaploid bread wheat (*Triticum asestivum*), three genes have been described that confer race non-specific resistance against multiple fungal diseases. These three genes, named *Lr34 (=Yr18/Sr57/Pm38), Lr67 (=Yr46/Sr55/Pm46)* and *Lr46 (=Yr29/Sr58/Pm39)* confer partial resistance against all races of the fungal diseases caused by wheat leaf rust (*Puccinia triticina*), stripe rust (*P. striiformis* f.sp. *tritici*), stem rust (*P. graminis* f.sp. *tritici*) and powdery mildew (*Blumeria graminis* f.sp. *tritici*) (Ellis *et al*. 2014, Spielmeyer *et al.* 2013). In addition, these genes are associated with a senescence-like phenotype referred to as leaf tip necrosis (LTN) (Krattinger *et al.* 2016, Risk *et al*. 2012). In wheat, LTN develops in the flag leaves at the heading stage under some environmental conditions (Singh and Huerta Espino, 1997). Two of these genes, *Lr67* and *Lr34,* have been cloned and respectively encode for a hexose transporter (Moore *et al*. 2015) and for a putative ATP-binding cassette (ABC) transporter (Krattinger *et al*. 2009, Dakouri *et al*. 2010). ABC transporters utilize ATP hydrolysis to translocate substrates across cellular membranes (Rea, 2007; Jasinski *et al.* 2003).

Multiple allelic variants have been found for *Lr34* and *Lr67* but only one resistance-conferring variant referred to as *Lr34res* and *Lr67res* has been found for each gene, respectively. Interestingly, both *Lr34res* and *Lr67res* are the result of rare mutation events that occurred after domestication in ancient wheat landraces (Kolmer *et al*. 2008, Krattinger *et al*. 2013, Moore *et al*. 2015). The LR34res protein version differs by only two amino acid polymorphisms from the LR34sus protein of which one is critical for disease resistance (Krattinger *et al*. 2009, Chauhan *et al*. 2015). Bread wheat was domesticated around 10,000 years ago, long after wheat shared its last common ancestor with other cereal species. An *Lr34*-like disease resistance has so far not been described in other globally important cereals like rice and maize. *Lr34res* was introduced into modern wheat in the 1900's by the Italian wheat breeder Nazareno Strampelli and has since been extensively used for crop protection (Kolmer *et al*. 2008). In particular, the International Maize and Wheat Improvement Center (CIMMYT) continues to use combinations of partial rust resistance genes with additive effects in its rust improvement program (Singh *et al.* 1991; Singh *et al.* 2005).

Orthologous *Lr34* genes are present in rice and sorghum but not in maize and in barley (Krattinger *et al*. 2011, 2013). However, only the susceptible haplotype has been found among these *Lr34* orthologs which is in agreement with the very recent emergence of the *Lr34res* allele after wheat domestication. Despite the absence of the resistant *Lr34* haplotype in cereals other than wheat, *Lr34res* was functionally transferred into barley and rice where it provides resistance against the adapted barley pathogens barley leaf rust (P*uccinia hordei*) and barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) as well as the rice-specific fungus rice blast (*Magnaporthe oryzae*), respectively (Risk *et al.* 2013, Krattinger *et al*. 2016). In barley, the expression of *Lr34res* resulted in high levels of disease resistance at the seedling stage. This was however accompanied by a strong LTN phenotype which had a negative impact on plant growth and yield (Risk *et al*. 2013, Chauhan *et al*. 2015). In rice, one of the transgenic lines showed a late LTN phenotype resulting in a plant development similar to the non-transgenic sib line but increased levels of rice blast resistance (Krattinger *et al*. 2016).

In this study, we transformed the Hi-II hybrid maize line with *Lr34res* driven by its native promoter. *Lr34res*-expressing maize lines showed an increased resistance against two of the most important foliar maize diseases, common rust and northern corn leaf blight.

(2) Results

2.1 – *Lr34res* confers common rust and northern corn leaf blight resistance in maize

Fifty-five independent primary transgenic (T0) plants were generated in the maize hybrid Hi-II by Agrobacterium-mediated transformation. From these 55 T0 plants, only 13 produced T1 kernels, while 12 did not survive and 30 were sterile. DNA blot analyses indicated that the 13 fertile T0 plants carry at least two insertions of the *Lr34res* transgene (1 line had 2 copies, 7 lines had 3 copies, and 5 lines had more than 3 copies).

Three independent transgenic events, called 161, 163 and 164, respectively, were selected for further characterization. Each of them carried three co-segregating copies of *Lr34res* (Figure S1b) and they were chosen based on the development of a LTN phenotype and on the number of available T1 kernels. *Lr34res* and sib T1 plants were advanced to the T2 generation and homozygous T2 plants for events 163 and 164 and segregating T2 plants for event 161 were

selected for further analyses. For event 161, all plants were analysed by PCR for the presence or absence of *Lr34res*.

Pathogenicity analyses were undertaken on these three independent T2 families using the biotrophic fungus common rust and the hemi-biotrophic fungus NCLB. For common rust, all three lines showed markedly increased resistance compared to their sibs lacking *Lr34res*. The plants derived from events 163 and 164 did not develop macroscopic symptoms 12 days after infection (d.a.i.), whereas their azygotic counterparts showed brownish pustules typical for common rust. A few and smaller pustules were visible on *Lr34res*-expressing plants of event 161 (Figure 1a, Figure S2). We quantified fungal biomass in infected plant tissue using fluorescently labelled wheat germ agglutinin (WGA-FITC) that specifically binds to the fungal cell wall component chitin (Ayliffe *et al*. 2013). Similar to the macroscopic observations, the *Lr34res*-expressing maize plants of all three independent events analysed in detail had significantly reduced chitin levels compared to their azygous siblings (Figure 1b). Chitin levels in event 161 were higher (0.93 µg / mg fresh weight) compared to plants of events 163 (0.10 µg) and 164 (0.089 µg), which is in agreement with the weaker macroscopic resistance phenotype in the former. However, even 161-derived transgenics had much reduced chitin levels compared with their azygous siblings lacking *Lr34res* (2.67 µg – 3.41 µg).

NCLB symptoms were evaluated macroscopically 10 and 14 d.a.i.. *Lr34res*-expressing maize plants showed a delay in symptom development with smaller lesions compared to the azyous siblings. At ten d.a.i., most of the *Lr34res* plants did not show visible signs of infection, whereas most of the sib plants already showed severe symptoms (Figure 1c). After 14 days, symptoms also appeared on *Lr34res* plants but they were less severe than on sib lines (Figure S3). Disease severity was also quantitatively assessed by scoring the percentage of plants showing symptoms from 8 to 13 d.a.i. and the area under the disease progress curve (AUDPC) was calculated according to Hurni *et al*. (2015). This quantification again revealed a delay in NCLB development in plants of the three *Lr34res*-expressing T2 families (Figure 1d). Our infection experiments showed that *Lr34res* confers a reduction in *P. sorghi* and *E. turcicum* development. The partial resistance phenotype is reminiscent of *Lr34res*' partial resistance phenotype in wheat.

2.2 – *Lr34res* expression in maize results in LTN but no apparent growth reduction

In barley and in rice, high *Lr34res* expression levels at the seedling stage were associated with a strong LTN phenotype resulting in a negative impact on plant development (Risk, *et al*. 2013; Krattinger, *et al.* 2016). Among four transgenic rice events, one showed low *Lr34res* seedling expression levels coupled with late LTN development and no obvious growth penalty (Krattinger *et al*. 2016). All three transgenic maize events developed a LTN phenotype appearing after ~6 weeks (Figure 2a). At the seedling stage, when the expression level was measured and the infection tests were performed, no LTN was visible. Hence, the *Lr34res* – based resistance was apparent before LTN emerged.

Seedling expression levels of the three independent maize events were assessed by RT-qPCR. Interestingly, plants derived from events 163 and 164 had 774-fold and 816-fold higher *Lr34res* expression levels compared to those of event 161 (Figure 2b). As shown above, despite the relatively low *Lr34res* expression levels in event 161, it showed a clear NCLB and common rust resistance phenotype. In order to determine a possible negative impact of *Lr34res* on plant development, we measured two parameters: plant height and above-ground fresh weight of *Lr34res*-expressing maize plants and respective sibs grown under greenhouse conditions (Figure 3). These parameters appear to be good indicators of potential negative impacts of *Lr34res* on plant development as they show an obvious reduction in *Lr34res*-expressing barley plants and the high-expressing rice lines (Risk *et al*. 2013; Krattinger *et al*. 2016). In *Lr34res*-expressing maize plants however, no significant differences were observed for these parameters compared with the respective sib lines. Also, visually there was no apparent impact on the plant development when *Lr34res* was expressed. These results indicate that unlike barley and rice, even a relatively strong expression of *Lr34res* as found in events 163 and 164 does not impact plant growth.

2.3 – Orthologous Lr34 genes in maize and distant relatives

Based on a comparative analysis, Krattinger *et al*. (2011) showed that the most similar maize homologue of the LR34 protein, GRMZM2G014282, was only 58 % identical to LR34res. In contrast, rice and sorghum have more similar LR34 orthologs with 86 % and 72 % amino acid identity, respectively. In order to test for the presence of *Lr34* orthologs in the *Zea* lineage (maize)

and its sister taxa, the *Tripsacum* lineage, a PCR probe was developed based on sequences that are conserved among *Lr34* sequences of wheat, rice and sorghum. This PCR probe spanned the critical sequence polymorphisms that distinguish *Lr34res* from *Lr34sus*. PCR was performed on genomic DNA of bread wheat (cv. Chinese Spring), rice (cv. Nipponbare), maize (Hi-II) and 10 different *Tripsacum* species (Table S1). Wheat, rice and *Tripsacum* showed PCR amplification of the expected fragment size, but no amplification product was produced from maize (Figure S4a). The amplification products of *Tripsacum* were sequenced and showed greater than 72 % nucleotide identity compared to the *Lr34* wheat sequence. The corresponding fragment of the rice and sorghum orthologs, *OsABCG50* and *Sb01g016775,* showed 90 % and 77 % nucleotide identity respectively, whereas no significant blast of this segment was obtained against the maize homolog GRMZM2G014282. All the *Tripsacum* sequences clustered within the '*Lr34* clade' in a phylogenic tree (Figure S4b) and all the *Tripsacum* species had the susceptible haplotype for the two critical sequence polymorphisms.

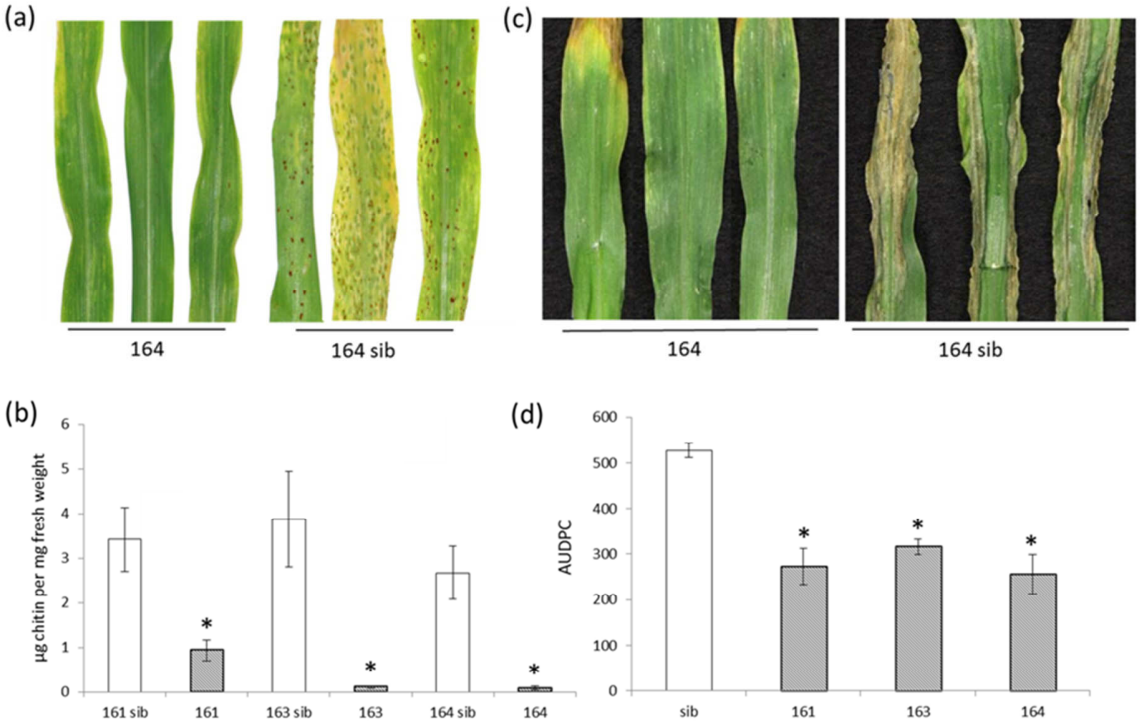

Figure 1. *Lr34res* provides partial resistance against common rust and NCLB in maize. (a) Macroscopic evaluation of common rust symptoms on seedling leaves of *Lr34res* transgenic maize event 164 and corresponding sib plant 12 days after infection. (b) WGA-FITC chitin quantification of common rust, 12 d.a.i. * indicates significant differences between *Lr34res*-expressing plant and an azygous segregant (Mann Whitney Wilcoxon test W=25, $p < 0.05$). Errors bars represent standard errors of 5 biological replicates. (c) Macroscopic observation of NCLB symptoms 10 d.a.i on *Lr34res* event 164 and its corresponding sib line. (d) Area under the disease progress curve (AUDPC) of the different *Lr34res* transgenic individuals and azygous sibling (pool of sib derived from the different events) calculated between day 8 to day 13 after infection. * indicates a significant differences to the sibs (Mann Whitney Wilcoxon test, W=18 or 24, $p < 0.05$).

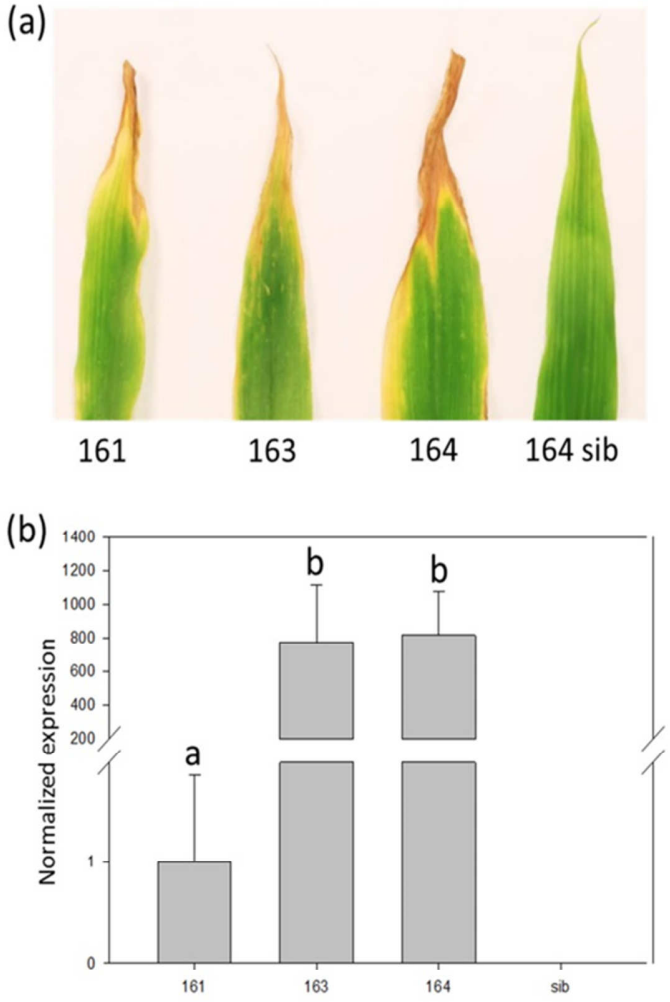

Figure 2. *Lr34res* results in a LTN phenotype in the three transgenic lines. (a) Representative example of the third leaf of six week-old seedlings showing a characteristic LTN phenotype. (b) Normalized *Lr34res* expression in transgenic lines. Data are normalized to the reference gene folylpolyglutamate synthase (FPGS). *Lr34res* expression level of line 161 =1. Letters indicate line with equivalent expression level (Mann Whitney- Wilcoxon *test,* $p < 0.05$). Errors bars represent standard errors from three biological replicates.

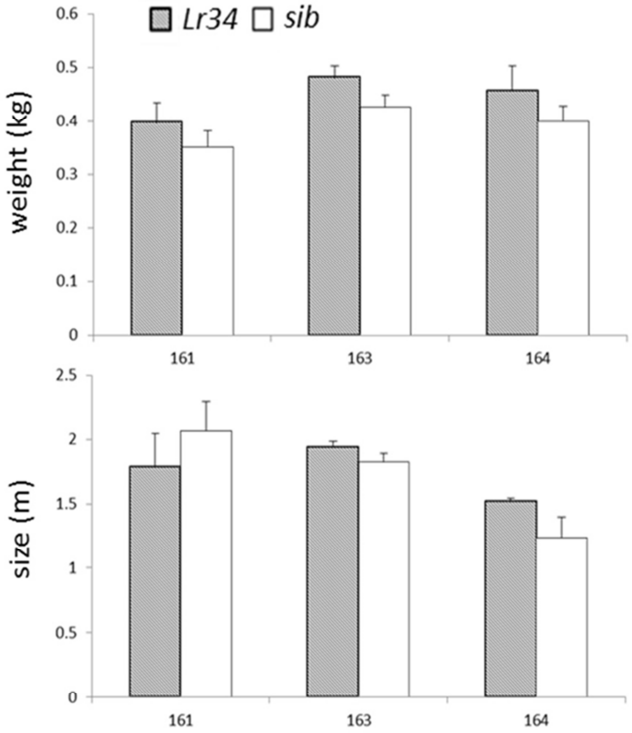

Figure 3. Measurement of growth parameters in *Lr34res* maize plants. Plant fresh weight in kg and plant height in m was assessed for the different plants and corresponding azygous siblings using at least 3 biological replicates of 14 weeks old plants. Statistical analysis was made using the Mann-Whitney Wilcoxon test and were always $p < 0.05$. Errors bars represent standard errors from the 3 to 7 biological replicates.

(3) Discussion

In this study, we showed that the wheat *Lr34res* allele is functionally transferable into maize and confers resistance against two maize diseases of agronomic importance: the biotrophic common maize rust pathogen and the hemi-biotrophic fungus that causes northern corn leaf blight disease. Hence, *Lr34res* can be effective against biotrophic and hemi-biotrophic diseases in the same crop species. *Lr34res* has already been shown to be effective against the hemi-biotrophic fungus rice blast in rice (Krattinger *et al*. 2016) and various biotrophic fungi such as rust and powdery mildew pathogens in wheat and barley (Risk *et al.* 2012; Risk *et al.* 2013). Furthermore, *Lr34res* has been associated with resistance against the hemi-biotrophic fungus spot blotch (*Bipolaros sorokiniana*) in wheat (Lillemo *et al.* 2013).

The infection processes of these fungi involve several steps, including pre-penetration, penetration, colonization and sporulation (Tucker *et al.* 2001). Each fungus uses a different mechanism to invade its host plant. Penetration can be direct, through the cuticle and the different layers of the cell wall, as this is the case for rice blast, NCLB and powdery mildew pathogens (Pederson *et al.* 2012; Haugaard *et al*. 2002; Zabka *et al*. 2008; Zhang *et al*. 2014; Wathaneeyawech *et al.* 2015; Knox-Davies 1974). Other pathogens like the rusts use natural openings such as stomata on the leaf surface (Song *et al.* 2011). Once inside the leaf, the obligate biotrophic rust and mildew fungi produce specialized feeding structures called haustoria for nutrient uptake (Song *et al*. 2011; Pederson *et al*. 2012; Haugaard *et al*. 2002; Zabka *et al*. 2008). The hemi-biotrophic fungi rice blast and NCLB also develop feeding vesicles by differentiation of primary hyphae (Wilson *et al*. 2012; Kankanala *et al.* 2007) that are very similar to haustoria and that are also surrounded by the host membrane (Wilson and Talbot, 2009; Muiru *et al*. 2008; Levy and Cohen 1983). In contrast to the biotrophic pathogens however, rice blast and NCLB switch to a necrotrophic growth phase after about 2-3 days (Marcel *et al.* 2010; Levy and Cohen; 1983). The development of a feeding structure for nutrient uptake is a commonality among all pathogens *Lr34res* is effective against. Although the exact molecular function of this gene is still unknown, it is likely that the LR34res protein restricts fungal growth during the biotrophic growth phase.

Qualitative and quantitative NCLB resistance genes have been identified in maize, for example the race-specific *Ht1* gene (Welz and Geiger 2000) and the quantitative gene *Htn1* that confers resistance against the most prevalent NCLB races (Hurni *et al*. 2015). *Htn1* encodes a putative wall-

associated receptor-like kinase that might be involved in the perception of an apoplastic signature of *E. turcicum* (Hurni *et al*. 2015). Common rust resistance genes have been mapped to 8 different loci (Rp1, 3, 4, 5, 6, 7, 8 and 9) (Hulbert 1997). *Rp1* and *Rp3* are the best characterized loci and each consist of a cluster of genes that encode intracellular nucleotide-binding, leucine-rich repeat receptor (NLR) proteins (Smith *et al.* 2010; Webb *et al*. 2002). Gene number at the *Rp1* locus differs considerably among different maize cultivars and can vary from one gene in some haplotypes to more than 50 paralogous genes in others (Smith *et al.* 2004). Within four maize lines (*HRp1-B*, *HRp1-M*, *MH95* and *B73*) a total of 61 *Rp1* genes, with a large diversity in the LRR region, were identified. Among them, thirty-two were transcribed and each of these genes can be distinguished based on the *P. sorghi* isolate to which it confers resistance (Chavan *et al.* 2015). An example is the *Rp1-D* gene, which has been successfully used in North America between 1985 and 2000. During autumn 1999, however, the emergence of a new *Rp1-D*-virulent rust population infected more than 40 % of the *Rp1-D* "resistant hybrids" (Pataki *et al.* 2001). This demonstrates that disease resistance based on single NLR genes is prone to breakdown because of the emergence and rapid spread of new pathogen strains. In contrast, *Lr34res* is a durable resistance gene with no pathogen adaptation observed so far. Hence, this gene might be useful in maize disease resistance breeding.

Orthologs of *Lr34* are found in some cereals, including rice with OsABCG50 (86 % amino acid identity) and sorghum with Sb01g016775 (74 % amino acid identity) and Sb01g016770 (72 % amino acid identity) (Krattinger *et al*. 2011). Members of the grass genus *Tripsacum,* a distant relative of *Zea,* also carry *an Lr34* ortholog. Maize does not have an ortholog of *Lr34* but can be crossed with *Tripsacum*. As shown by James in 1979, crosses between maize and various *Tripsacum* species result in pollen-sterile hybrids but produce kernels after backcrossing with maize. Maize x *Tripsacum* hybrids were already used in maize breeding programs for the introgression of traits of interest, like fatty acid composition (Duvick *et al*. 2006) or rootworm resistance (Prischmann *et al*. 2009). The *Tripsacum* accessions tested in this study all had the susceptible *Lr34* haplotype. However, *Lr34res* in wheat was the result of rare mutation events. Hence, it is possible that *Lr34* orthologs of the resistant haplotype might be found in a large *Tripsacum* collection at low frequency. Such rare variants could be crossed into the modern maize genepool by *Tripsacum* x maize hybridizations.

If no resistant *Lr34* variants can be found, genome editing could be used to transform the susceptible into resistant haplotype.

Interspecies gene transfer is a very powerful tool to move resistances between species and can thereby result in a broader resistance spectrum. For example, the NLR gene *Rxo1* has been successfully transferred from maize, where it provides resistance against diverse bacterial pathogens, such as *Xanthomonas oryzae* pv. *oryzicola*, the causal agent of bacterial streak disease, to rice (Zhao *et al.* 2005). Transgenic wheat expressing the Arabidopsis EFR receptor-like kinase was more resistant against the bacterial disease *Pseudomonas syringe* pv. *oryzae.* EFR perceives the highly conserved bacterial elongation factor Ef-Tu (Schoonbeek *et al*. 2015). Similarly, the rice receptor-like kinase (RLK) gene *Xa21* has been shown to provide Banana Xanthomonas wilt resistance in banana (Tripathi *et al*. 2014). The non-host resistance NHR-linked Arabidopsis genes *PING 4* (coding for a phospholipase-like protein (EARLI4-like)), *PING* 5 (coding for a leucine-rich repeat protein kinase) and *PING* 6 (coding for an ankyrin repeat family protein), known to provide pre-invasion resistance to non-adapted fungal pathogens, have been transferred into soybean where they confer Asian soybean rust resistance (Langenbach *et al.* 2016). *Lr34res* also has been successfully transferred in other crop species such as barley and rice (Risk *et al*. 2013, Krattinger *et al*. 2016). The fact that this gene functions across different monocots indicates that the mechanism of resistance is conserved and that the substrate transported by the ABC-transporter protein is likely shared among them.

It is known that expression of resistance genes often results in lower shoot biomass and negative effects (Burdon and Thrall. 2003). *Lr34res* is also known to negatively impact plant development in barley (Chauhan *et al* 2015; Risk *et al*. 2013) and in rice, where it was however possible to balance the fitness cost by modulating the gene expression level at seedling stage (Krattinger *et al*. 2016). From our greenhouse experiments, even under high *Lr34res* expression levels, the development of *Lr34res* expressing maize plants was not negatively affected. Whether *Lr34res* in maize does result in a small yield penalty or not can only be determined in field experiments.

To conclude, these results are a proof of concept that *Lr34res* is functionally transferrable into the globally most produced cereal crop, maize. Hence, this resistance gene is functional in all major cereals tested. In contrast to wheat and rice however, transgenic maize plants are already commercially grown and more than 30 % of the maize area was planted with GM plants in 2014

(Lucht 2015). Besides a transgenic approach, a detailed analysis of the *Lr34* ortholog in *Tripsacum* could offer a non-transgenic approach to establish *Lr34*-like disease resistance in maize.

(4) Experimental procedures

4.1 – Maize transformation and plant characterization

The genomic *Lr34res* construct under the wheat native promoter and terminator in the binary vector p6U (*p6U:gLr34res*; Risk *et al*. 2012) was transformed into the maize Hi-II (A188 x B73) hybrid (Horn *et al*, 2006) using *Agrobacterium tumefaciens*-mediated transformation according to a protocol previously established (Hensel et al. 2009, Van der Linde *et al*. 2012). Fifty-five T0-plants were recovered and the identification of the *Lr34res* transformants was done by PCR on genomic DNA using the *cssfr1* marker (Lagudah *et al*. 2009). The copy number was assessed by Southern blot using 7 µg of *Eco*RI-digested genomic DNA and a *hygromycin-phosphotransferase* (Ubi-HPT) ^{32}P-labelled probe (Figure S1a) (Risk *et al.* 2013). Plants were self-pollinated, resulting in T1 kernels.

4.2 – Maize rust infection

Three-week-old maize seedlings were grown in Jiffy pots (Ø 8 cm) with standard soil (Classic ED 73) in a growth cabinet under diurnal conditions with 16 hours light at 20°C and 8 hours dark at 18°C. Plants were then spray-infected with a *P. sorghi* isolate that was collected from a natural greenhouse infection on maize cultivar *sweet nugget.* Plants were sprayed using an air-assisted sprayer, with a solution of uredinospores (incubated at 40°C for 5 minutes in order to break the dormancy) and oil (Fluorinert FC-43). After inoculation, infected plants were incubated in the dark under high humidity (~95%) at 16°C for 24 hours. Then, plants were shifted to diurnal conditions (16h light /20°C, 8h dark /16°C, 70% humidity). Disease symptoms were assessed macroscopically for the presence of pustules on the leave surface and using chitin quantification 12 days after infection. Quantification of chitin was performed as described in Ayliffe *et al*. (2013). Five biological replicates of plant leaf tissue were harvested, weighed, cut into 3-cm fragments, and placed into 50-ml Falcon tubes. Sufficient volume of 1 M KOH containing 0.1% (vol / vol) Silwet L-77 (Lehle Seeds, Round Rock, TX, U.S.A.) was added to each tube to entirely cover the tissues. Tissues were then heated in a steam cooker for 20 minutes before being washed with 50 ml of 50

mM Tris, pH 7.0 and resuspended in a 50 ml volume of Tris. Plant tissues were macerated by sonication to generate a fine and uniform suspension. 200 µL of 1 into 10 water diluted samples were added to 10 µl of a 1-mg/ml solution of WGA-FITC before being measured using the fluorometer Synergy H1 Hybrid Reader (Biotek). The readouts were analyzed using the software Gen5, version 2.03.1 (Biotek).

4.3 – NCLB cultivation and infection

The northern corn leaf blight (*E. turcicum*) isolate passau-1 described by Hurni *et al.* 2015 was grown *in vitro* for three weeks on potato dextrose agar (PDA) (19.5 g / 500mL of ddH_2O) plates in the dark, upside down, at room temperature. Spores were harvested from plates by rinsing with 0.1 % tween 20 (Carl Roth) in sterile water and scraped with a spatula and filtered through a fine mesh (0.5 mm). Final density was adjusted to $4.5 * 10^4$ spores per ml, using a Neubauer counting chamber and sprayed on the three-week-old plants. Maize plants were grown for three weeks in Jiffy pots with standard soil (Classic ED 73) (Einheitserde), 15 plants in the same tray, in the greenhouse with cycles of 16 hours at 20°C with light and 8 hours at 18°C in the dark. Once the second leaf was fully expanded, the newly emerged young leaves were removed by cutting until the end of the experiment as described in Hurni *et al*. (2015).

Infected plants were kept under the same conditions but covered by plastic hoods in order to maintain a high humidity. First symptoms were observed 8 d.a.i. and were then recorded every day for 5 days. Disease severity was calculated as previously described by Hurni *et al*. (2015) and Madden *et al*. (2007).

4.4 – Quantitative real-time PCR analysis of Lr34res expression

Expression of *Lr34res* was quantified using RT-qPCR. Total RNA was extracted from three-week-old seedling leaves using the SV Total RNA Isolation System (Promega). RNA was quantified using a Nanodrop ND-1000 spectrophotometer and RNA integrity was checked on a 0.8% agarose gel in 1x sodium-borate (SB) buffer. The cDNA synthesis was then performed from 500 ng of RNA using the iScript™ advanced cDNA synthesis kit (Bio-Rad).

Expression of *Lr34res* was determined by RT-qPCR in a 10 µL volume reaction including 5 µL of KAPA SYBR® fast qPCR master mix (KAPA Biosystems), forward and reverse *Lr34res* qPCR primer (Risk *et al*. 2012) (500 nM final concentration), and 4 µL of 1:20 diluted cDNA. Samples were run on a CFX96 Touch Real-time PCR (Bio-Rad). Folylpolyglutamate synthase (FPGS) was used as

reference gene with the primers Forward 5' ATCTCGTTGGGGATGTCTTG 3' Reverse 5' AGCACCGTTCAAATGTCTCC 3' (Manoli *et al*. 2012).

4.5 – Determination of yield parameters under greenhouse conditions

At least three plants per transformation event were kept until adult stage (14 weeks) in a greenhouse under LED light. The greenhouse conditions were 60% humidity and 25°C. Plants were grown in standard soil (Classic ED 73) (Einheitserde). Fourteen-week-old plants were cut at soil level to determine fresh weight and plant height to the apex. Tassels were cut at their base and therefore excluded from the measure.

4.6 – Identification of Lr34 orthologous genes in maize and Tripsacum

PCR was performed on genomic DNA using primer set *Lr34_conserved_f2* (5'-CCATGGCCCTCGAAATGAAG-3') and *Lr34_conserved_r2* (5'-GCTGGTATTGCATATGCCCA-3').
PCR reactions were performed in a 20 µL total volume using 1 µL of 40 ng / µL gDNA and 19 µL of PCR mix (Sigma buffer 1X (Sigma-Aldrich), dNTPs (0.125 mM), primer forward and reverse (0.5 mM) and Taq polymerase (Sigma-Aldrich) (0.25 µL of 5 U/ µL)).
PCR products (~ 755 bp) were purified using the GenElute Gel extraction kit (Sigma-Aldrich). Purified products were sequenced using 1 µL of purified PCR product, 0.80ul Big Dye / BrightDye Terminator Mix (Thermo-Fisher), 0.25 µl primer (5 pmol / µl) and 2 µL of 5X sequencing buffer in a total reaction volume of 10 µL. Sequencing was performed using an ABI-3730 Sanger sequencer (Applied Biosystems) and sequences were analyzed with FinchTV® software (Geospiza).
Sequence alignments and phylogenetic tree construction were performed using the web service phylogeny.fr (Dereeper *et al*. 2008; 2010). The multiple alignment was done with MUSCLE® software (Edgar *et al*. 2004), the alignment curation with GBlock® software (Castresana 2000, Talavera *et al*, 2007), the construction of the phylogenetic tree with PhyML® (Guindon *et al*, 2010) and the tree rendering with TreeDyn® (Chevenet *et al*. 2006).

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(7) Supplementary Figures

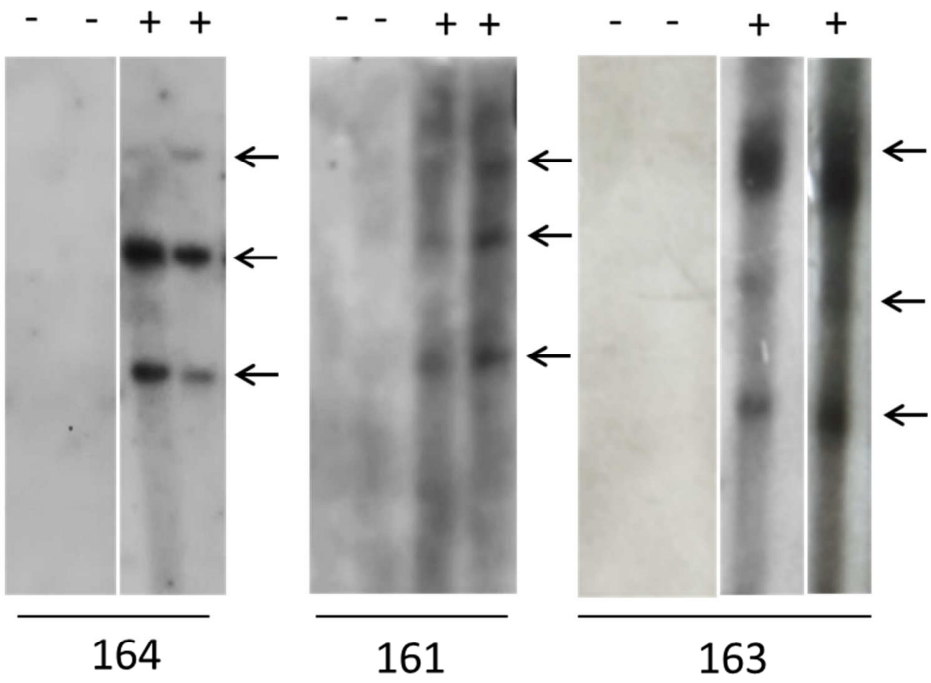


Figure S1. Southern Blot showing T-DNA copy number in the *Lr34res* transgenic maize. Events 161, 163 and 164 at the T2 generation are represented. + indicates plants with *Lr34res* and – segregating siblings without *Lr34res*.

Figure S2. Macroscopic common rust symptoms on plants derived from events 161 and 163 and corresponding sibs, 12 d.a.i. Infections were done at seedling stage on three-week-old plants.

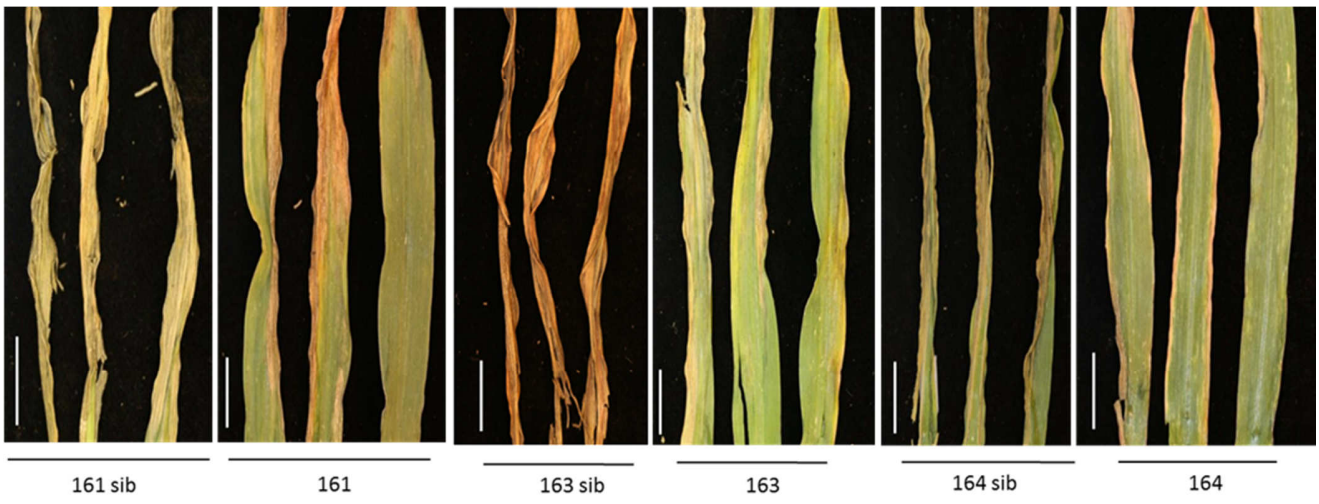
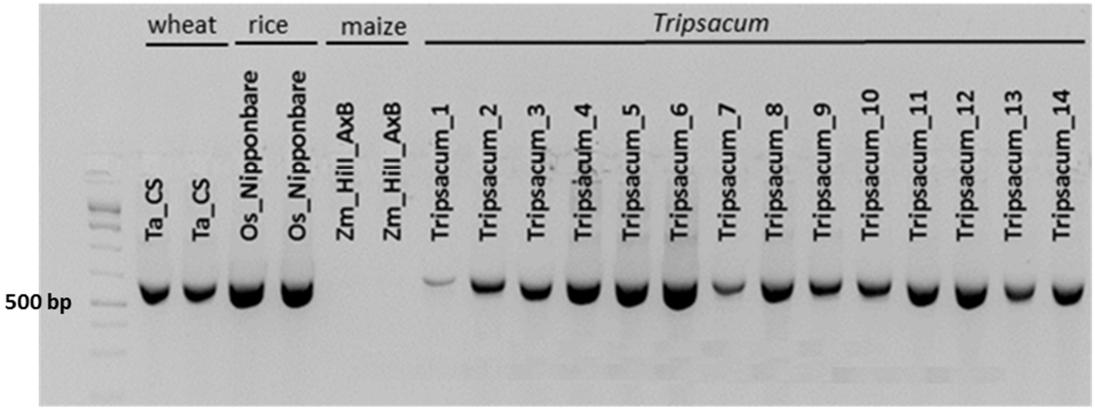


Figure S3. Macroscopic observation of NCLB symptoms on the different *Lr34res* transgenic maize plants and their corresponding azygous siblings 14 days after infection. Infections were done at seedling stage on three-week-old plants. Scale bar = 10 mm.

Table S1. List of the 10 T*ripsacum* species used for the study of the *Lr34* orthologous gene, with the name of the different species, their corresponding ID and accession numbers from the CIMMYT maize germplasm database and their country of origin .

Species Name	ID	ACC	Country
dactyloides	28224	25536	USA
dactyloides var.meridonale	28253	25565	VEN
cundinamarce	28254	25566	COL
andersonii	28262	25574	HND
jalapense	28305	25617	MEX
jalapense	28309	25621	MEX
australe	14651	12826	COL
peruvianum	28334	25646	PER
peruvianum	28335	25647	ECU
maizar	28356	25668	MEX
maizar	28357	25669	MEX
pilosum	28358	25670	MEX
lanceolatum	28360	25672	MEX

(a)



(b)

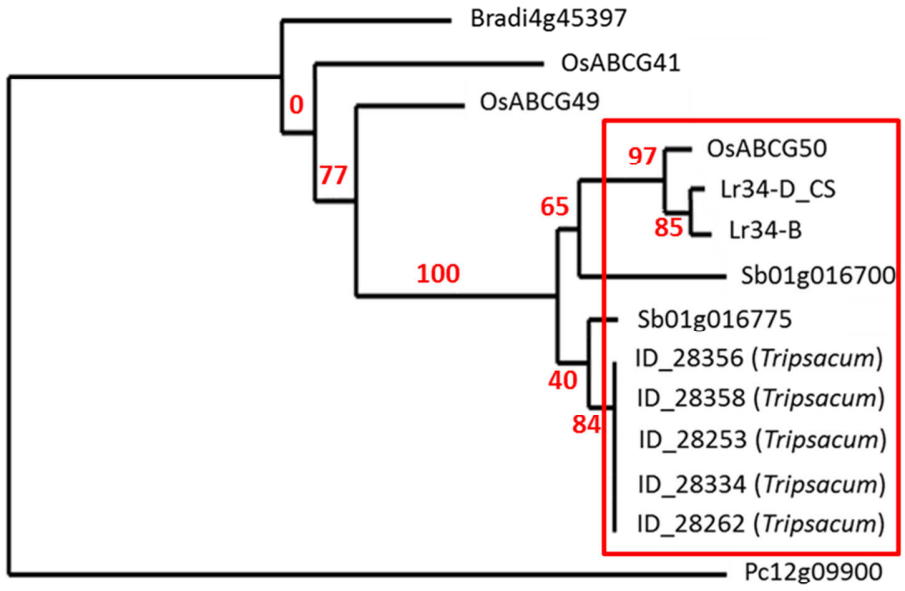


Figure S4. Study of orthologous *Lr34* genes in maize and its distant relative *tripsacum*. (a) Agarose gel showing genomic DNA amplification of *Lr34* on wheat (Chinese-spring), *OsABCG50* on rice (Nipponbare) and the *Lr34* orthologs on 10 different *tripsacum* species. No amplification was obtained for maize *Hi-II*. (b) Phylogenetic tree based on genomic DNA sequences of *Lr34*, of three of the most homologous rice genes sequences (*OsABCG50*, *OsABCG41* and *OsABCG49)*, two sorghum orthologous sequences (*Sb01g016775* and *Sb01g016700)*, one *Brachypodium distachyon* homolog (*Bradi4g45397)*, and 5 different *tripsacum* species. The "*Lr34* orthologous cluster" is marked in red. The ABC-like sequence from *Penicillium chrysogenum* (*Pc12g09900*) was used as outgroup to root the tree. Numbers indicate how many times the sequences to the right of the fork occurred in the same group out of 100 trees.

Chapter D

Analysis of the barley powdery mildew and wheat leaf rust transcriptomes during growth on susceptible and *Lr34* resistant plants

Justine Sucher, Fabrizio Menardo, Coraline Praz, Simon Krattinger and Beat Keller

Summary

Durable resistance against pathogens is highly valuable for disease management in agriculture and it is essential to understand the molecular mechanisms of such naturally existing resistance. Many studies on durable disease resistance in plants focused exclusively on the mechanisms on the host side. Studies on possible reactions and adaptations of pathogens exposed to durable resistance have been rare but are essential to understand the molecular basis of durability. The *Lr34* wheat gene, coding for a putative ABC-transporter, provides a broad-spectrum and durable resistance against multiple fungal pathogens in wheat, and it has been shown to also confer resistance to fungal pathogens in other crop species belonging to the grasses. *Lr34*-based resistance is partial and pathogens can grow and reproduce so some degree on *Lr34*-containing plants. This makes *Lr34*-expressing plants an ideal tool to study the response of pathogens to partial resistance. Here, we compared the transcriptomic response of the two pathogens leaf rust and powdery mildew during growth on wheat and barley, in the presence and absence of the *Lr34* resistance gene. Two different time points after infection were chosen for powdery mildew analysis on barley and one time point for the wheat leaf rust interaction. Transcriptome analyses revealed that there were no differences in the expression patterns of the two pathogens growing on susceptible *vs* partially resistant plants, reflecting the absence of an observable reaction of the pathogen to the *Lr34* gene. Thus, the durability of *Lr34* might be explained by the absence of a transcriptional reaction of biotrophic pathogens and, consequently, no adaptation to host defense.

(1) Introduction

Wheat, maize and rice are the most important crop species for food security, contributing about 90% of the cereals consumed (Ranum *et al.* 2014). Plant diseases cause more than 10% losses every year (Krattinger and Keller, 2016) and among them fungi represent the most important type of pathogens (Van Alfen 2001). Based on their life styles, fungal pathogens are classified in groups, called biotrophic, hemi-biotrophic and necrotrophic. All of them need to take up nutrients from their host in order to survive and grow (Urooj and Muthappa 2015), but they use different nutrient acquisition strategies. Biotrophic pathogens require living plant tissue to complete their infection cycle (Koek *et al.* 2011) whereas necrotrophic fungi actively kill their host as they colonize it (Laluk and Mengiste 2010). The hemi-biotrophic pathogens obtain nutrients in a first biotrophic phase on living plant tissue, before switching to a necrotophic phase at the late stage of the infection cycle, leading to the destruction of the host cells (Urooj and Muthappa 2015). Biotrophic pathogens acquire nutrients through specialized feeding structures called haustoria (Garnica *et al.* 2014). In contrast, necrotrophic fungi produce toxins, hydrolytic enzymes and necrosis-related proteins shortly after penetration in the host cells inducing plant cell membrane degradation (Divon and Fluhr. 2007). To establish an obligately parasitic interaction with their hosts, biotrophic pathogens manipulate the plant metabolism by the production of small secreted proteins (SSPs), including fungal effectors which are secreted by the haustorium (Szabo and Bushnell 2001; Garnica *et al.* 2014; Chen *et al.* 2013). Biotrophic and hemi-biotrophic fungi encode a larger number of these effector-like SSPs in their genomes than necrotrophic fungi (Kim *et al.* 2016). Some of these small secreted proteins can be recognized by a matching resistance protein of the host, leading to a disease resistance reaction induction, called effector triggered immunity (ETI) and an incompatible interaction between the host and the fungus (Bell *et al.* 1984; Jones and Dangl 2006). In most of the cases the resulting resistance is complete. If there is no recognition of fungal effectors by the plant there is no defense mechanism activation and the infection is successful leading to a compatible interaction (Gaudet *et al.* 2007). Two main types of compatible plant-pathogen interactions have been reported: the compatible or highly compatible interactions, when the plant is fully susceptible and the partially compatible interaction, when the plant is partially resistant (Lopez Kleine *et al.* 2012). Partial resistance represents a partially compatible interaction since the

fungus is still able, to some degree, to grow and colonize the plant tissue. The limites growth of the pathogen is due to gene expression in the host resulting in a modified host metabolism, allowing less growth of the pathogen (Vergne *et al.* 2010). Gene expression of the host and the fungal pathogen are dependent on the type of interaction (compatible or incompatible), the stage of infection (penetration, colonization, sporulation) and the pathogen life cycle (biotrophic or nectrophic) (Both *et al*. 2005; Coram *et al.* 2008).

In wheat, Coram *et al.* showed in 2008 that 115 transcripts were differentially expressed between compatible and incompatible interactions of stripe rust on *Yr5* resistant or *yr5* susceptible plants. Similar results were described in Wang *et al*. (2010) where 94 genes were preferentially induced during compatible interaction compared to incompatible interaction of wheat and stripe rust. Differential gene expression was also reported in rice between Nipponbare carrying the resistance gene *Pia* infected with the P91-15B isolate (compatible interaction) and infected with Ina86-137 (incompatible interaction). In contrast to other studies analyzing only host gene expression, this study also focused on the rice blast (*Magnaporthe oryzae*) pathogen gene expression and showed that genes were differentially expressed between the compatible and incompatible interaction (Kawahara *et al*. 2012). Among the differentially expressed fungal transcripts, a significant number encoded for putative secreted proteins. CSEPs (candidate secreted effector proteins) have also been shown to be significantly differently expressed between compatible and incompatible interaction of the biotrophic fungus barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) (*Bgh*) (Hacquard *et al.* 2013).

Differentially expressed genes on the host side have been shown in case of partial resistance (Darvishzadeh *et al.* 2008; Smart *et al.* 2003; Rubiales and Nicks. 1995) but no studies have been reported concerning gene expression in pathogens. The wheat gene *Lr34* is a partial, broad spectrum, multi-pathogen and durable resistance gene which has been used in breeding for a century and no pathogen adaptation has been observed so far (Krattinger *et al.* 2016). This gene has been successfully transferred into other crop species of global importance including barley and rice (Risk *et al.* 2013; Krattinger *et al.* 2016) and has been shown to confer partial resistance by slowing down the rust, mildew and blast infection process for all isolates tested so far. The aim of this study here was to investigate the pathogen gene expression profile during its growth on near-isogenic plant lines partially resistant based on the *Lr34* gene or the corresponding susceptible

genotypes in order to have a better understanding on the resistance mechanism. We performed RNA-sequencing of the two biotrophic fungal pathogens barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) (*Bgh*) and wheat leaf rust (*Puccinia triticina*), respectively on barley (cv. Golden Promise) and wheat (cv. Arina). For *Bgh*, two time points were selected, 48 hours after infection, corresponding to haustoria formation and 5 days after infection. Results showed that there was no difference in the pathogen transcriptome on partially *Lr34* resistant or susceptible plants. Thus, the durability of the wheat gene *Lr34* can be partly explained by this absence of fungal reaction to the resistance gene.

(2) Results

2.1- RNA-sequencing of barley infected with powdery mildew and wheat infected with leaf rust

Gene expression of the fungal pathogens barley powdery mildew and wheat leaf rust, growing either on *Lr34res* expressing plants or their corresponding sister lines (with no *Lr34* transgene) was studied by RNA-sequencing analysis. In transgenic barley, the *Lr34res* gene was under control of the inducible germin-like protein (GLP) promoter (Boni *et al*. unpublished). For wheat, the endogenous *Lr34res allele* was present. The studied barley and wheat genotypes showed no negative effects due to the presence of *Lr34res* (Boni *et al*. unpublished).

Two time points known to be important for the pathogen infection process were selected for mildew infection on barley: the colonization stage and haustorium formation, at 48 h.p.i (hours post infection) and the sporulation stage at 5 d.p.i (days post infection), when symptoms are visible on the leaf surface (Tremblay *et al.* 2013) (Figure S1.a.). The reads obtained by sequencing were mapped against the pathogen reference transcriptomes (Spanu *et al.* 2010, Xu *et al*. 2011). At 48 hours, between 1.19 % and 1.28 % of the total reads mapped to the *Bgh* reference genome assembly and at 5 days, between 21.71 % and 51.25 % of the total reads could be uniquely mapped against the *Bgh* reference sequence (Table 1a.). According to the *Bgh* reference transcriptome size which is 9 Mb (Spanu *et al.* 2010), the estimated coverage was about 10 x for the early time point and 72 x and 161 x for the later time point on *Lr34res* and sib respectively

(Table 1a). The very low percentage of reads mapped against the reference at 48 h.p.i was due to the low fungal biomass present in the leaf at this stage.

One time point at the late sporulation stage (Figure S1.b), was chosen for the analysis of the leaf rust transcriptome on wheat since it is difficult to control infection under natural field conditions. Between 41 and 53 million reads were obtained for the different samples. 36 % (19,437,144 reads) of the total reads were mapped uniquely against the leaf rust reference for Arina_*Lr34res* and 51 % (21,135,663 reads) for Arina, corresponding to 136 x and 147 x of fungal transcriptome coverage, according to the reference leaf rust transcriptome size of 18 Mb (Cuomo et al. 2016) (Table 1b.). These percentages of mapped reads against the fungal reference were comparable to data obtained with *Puccinia striiformis* at a similar infection time point (Dobon *et al.* 2016).

2.2 –The pathogen gene expression profiles are not changed in the presence of *Lr34res*

Of 7,431 annotated barley powdery mildew genes (Spanu *et al*. 2010), 5,638 genes (76 %) at 48 hours post infection (h.p.i) and 5,662 genes (76 %) at 5 days post infection (d.p.i) had at least one sequence read count in at least two of the three biological replicates. The expression level of all *Bgh* genes on *Lr34res* expressing plants or on sib lines was represented by plotting the expression average in rpkm (reads per Kb per million mapped reads) (Figure 1.). In order to identify differentially expressed genes (DEGs), a threshold of a $\log_2$ 1.5 and a p.value of 0.01 were chosen.

At the late sporulation stage, only five genes, among 5778, were found to be differentially expressed for *Puccinia triticina* on wheat. Four genes were up regulated on the resistant plants and one was down regulated (Figure S2.; Table S1.).

Similar results were observed for *Bgh* on barley at 5 d.p.i. where only four genes were differentially expressed between *Bgh* growing on *Lr34res*-expressing plants compared to sibs. The 18S ribosomal RNA and one gene encoding a CSEP were up-regulated in *Bgh* growing on *Lr34res* and one opsin like protein and an unknown conserved protein were down regulated with a $\log_2$ fold change higher than 1.5 (Figure 1.b, Table S2). Interestingly, at the earlier time point of *Bgh* infection, 48 h.p.i, 38 DEGs were identified. 19 were up-regulated and 19 were down-regulated in *Bgh* growing on *Lr34res* (Table S3.). Among them, 27 had a $\log_2$ FC lower than 2, corresponding to less than 4 fold change and the highest $\log_2$ fold change was 3.16. Furthermore the absolute

expression level of most of the differentially expressed genes was very low (< 10 rpkm) as shown by the distribution of the gene expression level (Figure 2.) which was four times lower for the 38 DEGs compared to the total gene set expression level. Due to the low fungal biomass in the leaf at 48 h.p.i, the fungal transcriptome coverage was very low (8X – 11X). RNA-seq artefacts can be introduced by low transcriptome coverage, resulting in a variation of the number of sequence reads (McIntyre *et al.* 2011; Ignatiadis *et al.* 2016). To verify the hypothesis that the low read numbers affected the number of DEGs, a coverage simulation was run. For this, fractions corresponding to 5%, , 20% and 50% of the 5 d.p.i time point total reads number were randomly selected (Figure 3., Table S3.). Five independent simulations were performed and showed that the number of DEGs increased with decreasing the coverage. The analysis performed on the 5% sample revealed ~ 22 DEGs, which is 5 times more than when using 100% of the reads (Figure 3.). Thus, our data indicate that a large number of the 38 DEGs at 48 h.p.i, compared to the 4 DEGs at 5 d.p.i, might be due to a low transcriptome coverage, which resulted in technical variation during the sequencing. We then tested gene expression of seven of these 38 DEGs by RT-qPCR.

2.3 - DEG validation by RT-qPCR

RT-qPCR analysis was shown to have a high correlation with RNA-seq data (Fang and Cui 2011) and is commonly used to validate the gene expression level (Bhargava *et al*. 2014). Among the top up and down regulated genes of *Bgh* at 48 h.p.i, seven were chosen for RT-qPCR validation. These genes were chosen according to their rpkm level from low expression (< 10 rpkm) to moderately expressing genes (> 100 rpkm) (Table 2.) (Figure S3, S4). The RT-qPCR analysis on seven differentially expressed genes (Figures 4.b, 5.b) based on the same RNA samples used for the RNA-seq experiment (Figures 4.a, 5.a) did not show significant expression differences. Thus, RNA-seq results were not reproduced by the RT-qPCR method. Furthermore, RT-qPCR using a new independent *Bgh* infection confirmed the results obtained and showed no significant differences of the pathogen growing on *Lr34res* plants compared to sibs (Figure 4.c, 5.c). We therefore conclude that no major changes, concerning the expression profile occurred in the pathogen on *Lr34res* plants.

Table 1. Percentage of reads mapped against the reference among the total reads number and transcriptome coverage. a) *Blumeria graminis f. sp hordei* and b) *Puccinia triticina* reference.
h.p.i: hours post infection d.p.i: days post infection.

a)

	T1 (48 h.p.i)		T2 (5 d.p.i.)	
	GLP_*Lr34res*	sib	GLP_*Lr34res*	sib
% mapped reads	1.19 ± 0.3	1.28 ± 0.2	21.71 ± 5.2	51.25 ± 4.4
mapped reads	808,049	632,785	5,159,307	11,529,965
total reads	60,799,426	49,101,075	23,910,585	22,535,753
Bgh coverage	11.3 X	8.85 X	72 X	161 X

b)

	High sporulation	
	Arina_*Lr34res*	Arina
% mapped reads	36.29 ± 3.7	51.89 ± 4.8
mapped reads	19,437,144	21,135,663
total reads	53,505,923	41,407,083
Bgh coverage	136 X	147 X

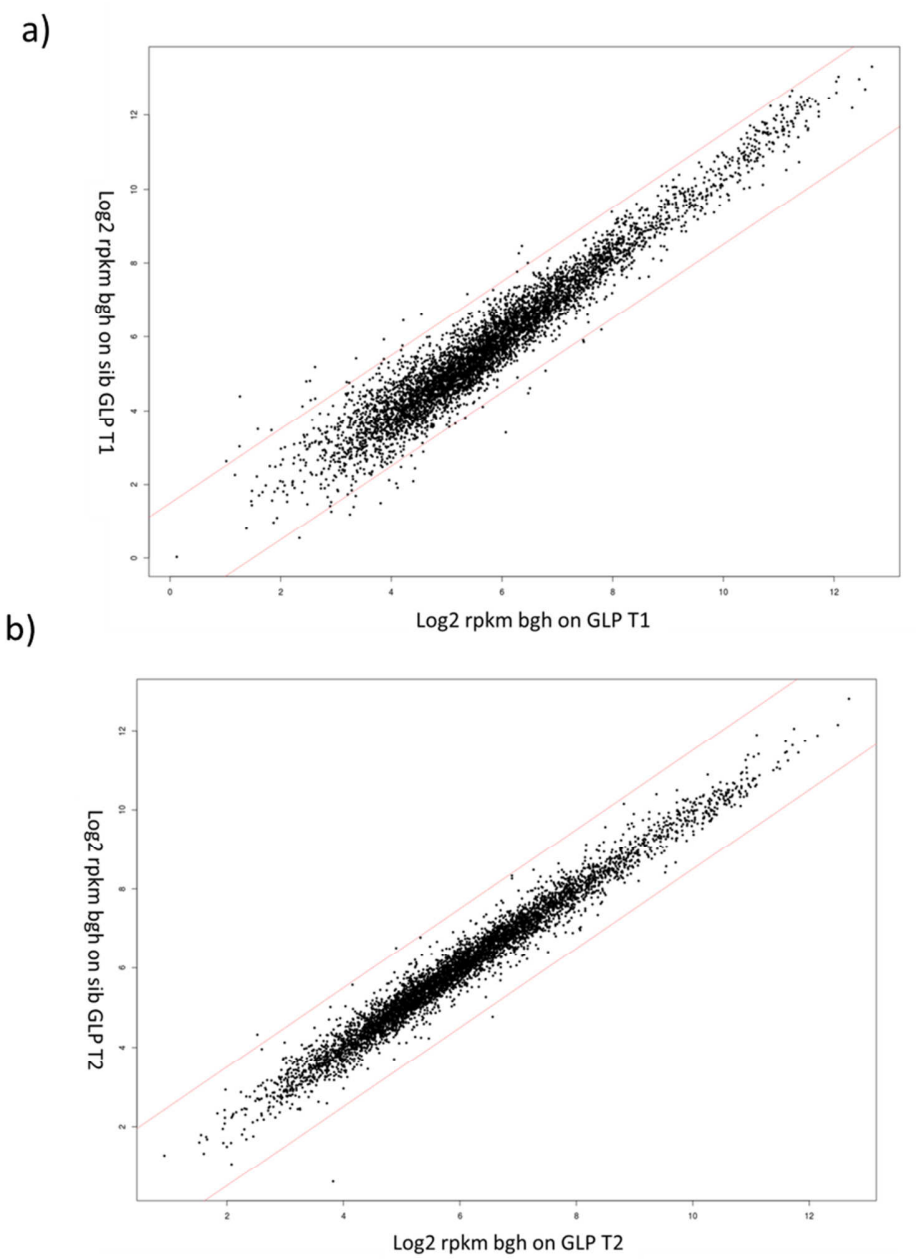


Figure 1. Plot of average changes in gene expression levels between *Bgh* growth on the two different hosts *Lr34res* and sib barley. The values are shown in log2 of the number of reads per Kb per million mapped reads (rpkm). The two red lines represent the threshold of two log1.5 fold change in expression (equal to a 3-fold change). The general *Bgh* expression is very similar between its growth on the two hosts and there are no genes that are highly differentially regulated. a) at 48 h.p.i, 38 genes were significantly differently expressed between the two conditions (fdr corrected p-value < 0.01) with more than a 3-fold change. b) at 5 d.p.i four genes were differentially expressed.

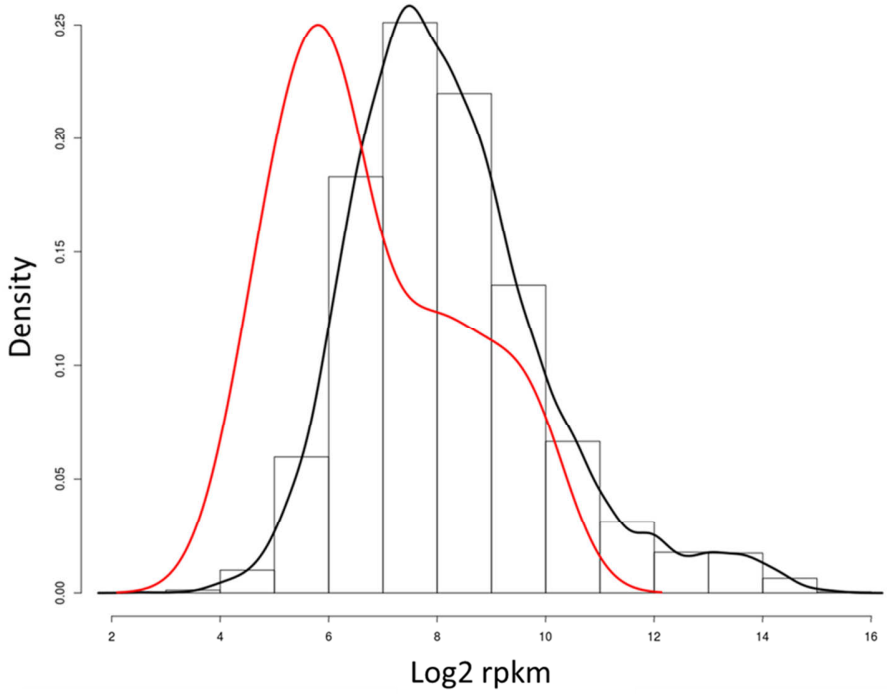


Figure 2. Gene expression distribution (log_2 rpkm). Histogram represents the entire gene set expression distribution of *Bgh* at T1 (48 h.p.i) for all the different biological replicates (the three GLP_*Lr34res* and the three sibs). The observed distribution of the entire gene set is represented by the black line and theobserved distribution of the 38 DEGs by the red line.

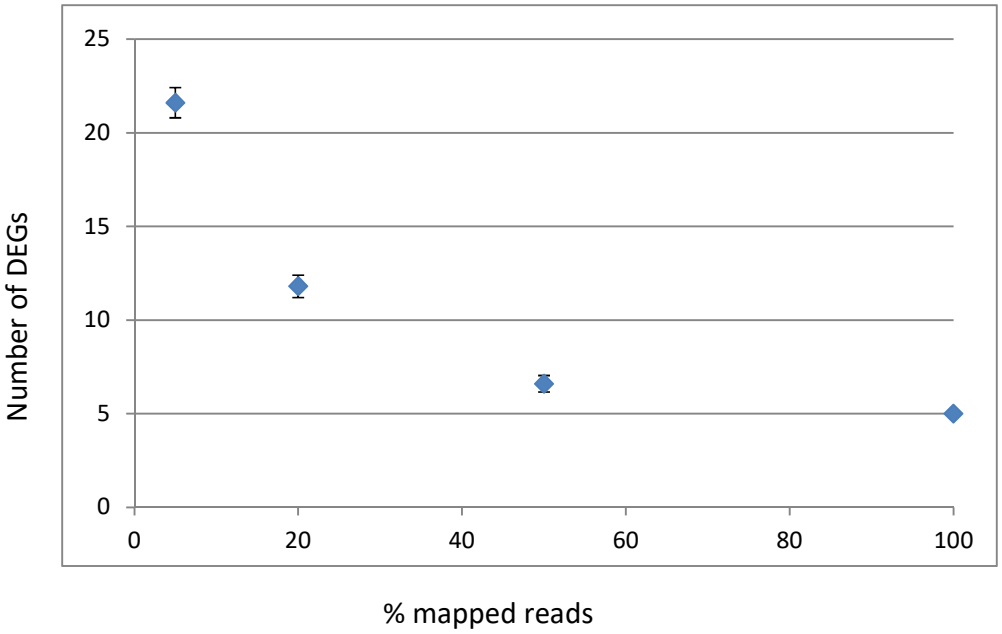


Figure 3. Simulation of the number of DEGs in relation to the number of sequence reads. The number of DEGs increased with lower coverage and/or smaller number of mapped reads. 100% represents all the reads which mapped against the *Bgh* transcriptome reference at T2 (5 d.p.i.) with a very good coverage of more than 72X. Results were based on five independent simulations. Error bars are standard errors.

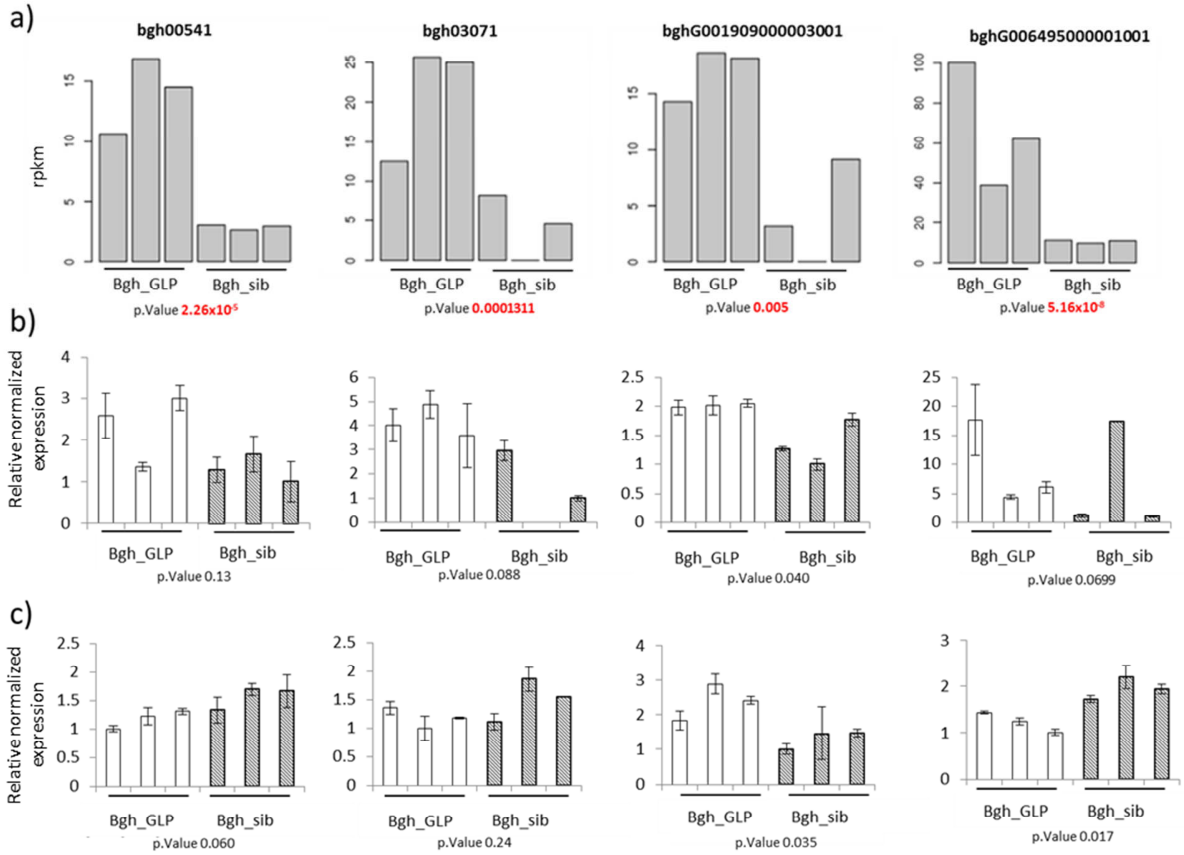


Figure 4. Four of the *Bgh* genes which showed up-regulation according to the RNA-seq analysis, at 48 h.p.i a) RNA-seq b) RT-qPCR using the same samples as for the RNA-seq experiment c) RT-qPCR using independant samples from a new *Bgh* infection. p. values are student t.test.

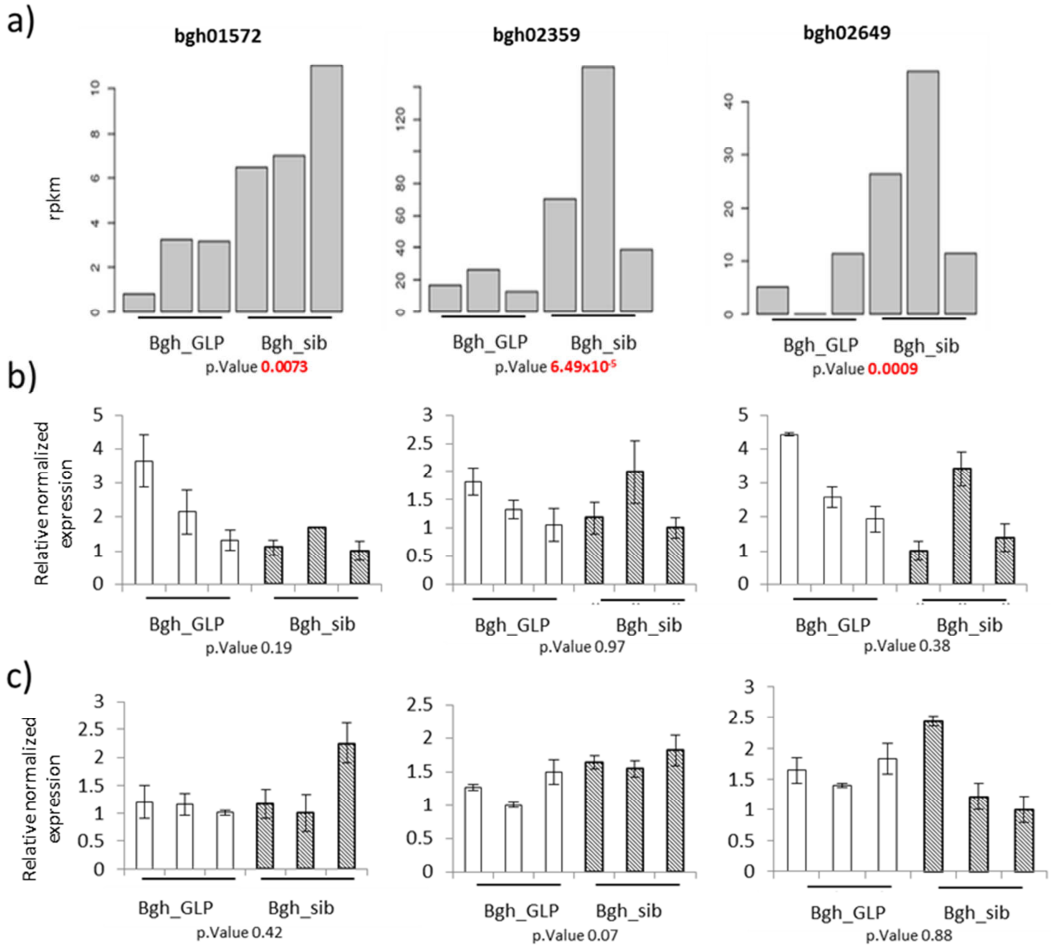


Figure 5. Three of the most down-regulated genes of *Bgh* at 48 h.p.i a) RNA-seq b) RT-qPCR using the same samples as for the RNA-seq experiment c) RT-qPCR using new samples from a new *Bgh* infection. p. values are student t.test.

(3) Discussion

The wheat *Lr34res* resistance gene slows down pathogen infection and confers partial resistance. It codes for a putative ABC-transporter (Krattinger *et al.* 2009) but its molecular mechanism remains unknown. Several transcriptomics studies on *Lr34* focused on the plant side and showed clear expression pattern differences between the *Lr34res* and susceptible plants. In 2007, Hulbert *et al.* showed that *Lr34res* wheat plants showed an up-regulation of genes associated with ABA inducibility and with osmotic and cold stresses. Transcriptomic analysis on transgenic *Lr34res* barley plants also showed strong differences in the expression pattern compared to the corresponding sib, with up-regulation of genes from multiple defense pathways mainly involved in basal and inducible disease resistance (Chauhan *et al*. 2015).

Many of the plant resistance genes involve a hypersensitive response (HR) that is based on the recognition of the corresponding pathogen *Avr* gene product and HR is a typical feature of gene for gene interactions (Jones and Dangl 2006; Yu et al. 1998). Rubiales and Niks (1995) showed that the resistance provided by *Lr34res* is not associated with a HR response. There was no induction of reactive oxygen species (ROS) production, of cell death or of callose deposition in wheat (Risk *et al.* 2012).

Microscopic observation of the rice blast growth on *Lr34res* plants showed that the slowdown of the infection process was sufficient to delay the disease development and reduce the symptoms (Krattinger *et al.* 2016). The resistance provided by *Lr34res* is quantitative and pathogens can still grow on the resistant host and sporulate. Therefore, it was possible to perform a transcriptomic analysis in order to compare the fungal gene expression pattern between growth on *Lr34* resistant or susceptible plants. There are only very few studies focusing on the pathogen transcriptome during infection but it is known that pathogens have different infection steps, involving different gene expression patterns (Tremblay *et al*. 2013; Pennington *et al*. 2016). It has been shown that the early stages of the *Bgh* infection process such as the germination and the appressoria formation are not fundamentally different in *Mla* barley compatible and incompatible interaction. The differences occur during the later stages of the infection with the haustoria formation and the establishment of secondary hyphae (Caldo *et al.* 2004). In *Blumeria graminis* on compatible or

incompatible host most of the differentially expressed genes were CSEPs (candidate secreted effector proteins) belonging to the haustorium formation cluster family (Haquard *et al*. 2013).

In this study, we investigated pathogen expression and performed a transcriptomic analysis using the two fungal pathogens, *Blumeria graminis* f. sp *hordei* and *Puccinia triticina* on *Lr34* resistant and susceptible plants. The two pathogens did not respond to the presence of the *Lr34* resistance gene in the plant. The gene expression analysis of powdery mildew at 48 h.p.i and 5 d.p.i, as well as the leaf rust sporulation stage analysis showed that the pathogen transcriptomes were highly similar, with no major changes between their respective growths on *Lr34* resistant or susceptible plants.

These results, together with the absence of HR, suggest that the *Lr34* resistance might be due to a more hostile environment for the pathogen because of the plant defense genes up-regulation which limits the pathogen growth. Thus, the selection pressure is lower than in the case of gene for gene interaction and would be an explanation of *Lr34res* durability. Indeed, *Lr34res* is used in plant breeding since more than 100 years and no pathogen adaptation has been reported so far (Kolmer *et al.* 2008). To understand partial durable resistance, it is essential to also understand the pathogen side. Here we showed that no major changes occurred in pathogen expression profile when growing on *Lr34* resistant plants.

(4) Experimental procedure

4.1 - Pathogen infections

Two weeks old barley plants were inoculated with the K1 powdery mildew isolate. Plants were maintained in an incubator under standard conditions until symptom development.

Leaf rust infection of wheat was performed in the field. A mixture of 16 Swiss isolates was used for the infection as described in Messmer *et al*. (2000). Inoculation was started by planting artificially infected plants into the spreader rows. Flag leaf samples were collected ~ 2.5 months after infection of the spreader rows and symptoms were clearly visible on the leaf surface.

4.2 - RNA extraction and sequencing

After 48 h.p.i and 5 d.p.i, pools of three barley leaves, from three different plants, were collected for each biological replicate. At high sporulation stage, infected wheat leaves were collected from the field. Pools of three flag leaves, from different location in the field, were collected for each biological replicates. Three biological replicates were collected for each condition. RNA was extracted using the miRNeasy kit (Qiagen), which allows the RNA extraction from the plants but also from the pathogen. RNA quality was assessed on a 0.8% agarose gel and concentration was measured using a nanoDrop ND1000 spectrophotometer and adjusted to 100 ng / µL. The Illumina sequencing Hi-seq 2000 of the 125 bp single ends RNA libraries was performed at the functional genomic center (Zurich).

4.3 - Transcriptomes analysis

RNA-seq reads were mapped against the reference transcriptome of *Blumeria graminis* f.sp. *hordei* (assembly EF1) (Spanu et al. 2010), for powdery mildew on barley and against the reference transcriptome of *Puccinia triticina* (assembly ASM15152v1) (Xu et al. 2011) for leaf rust on wheat, using the STAR guideline (Dobin et al. 2013). The set parameters were one mismatch allowed per 100 bp and no multi-mappers. Read counts were determined using featureCounts 1.4.6 (Liao et al. 2014) and statistical analysis was performed with edgeR package from R software. All expressed genes were tested for differential expression, between the two different conditions, with a general linear model and a tagwise estimation of dispersion (Robinson et al. 2010). The threshold selected for differentially expressed genes was log fold-change of 1.5, corresponding to a three folds difference of expression and a p.value < 0.01. For the coverage simulation, a random subset of 5%, 20% and 50% of the 5 d.p.i total raw reads were selected prior the sequence analysis.

4.4 - RT-qPCR validation on differentially expressed genes

A set of seven genes with the greatest differential expression (up-regulated and down-regulated) between GLP_*Lr34* and sib at 48 hours after *Bgh* infection and with various rpkm level (from low < 10 to high > 100) were selected for the RT-qPCR validation. Primer sets were designed for all the

seven selected genes and blasted against the barley and the *Bgh* genomes to be sure that amplification was gene specific. A standard curve to assess primers efficiency was performed (Table S5). cDNA from the different RNA samples was synthetized from 1 µg of RNA, using the i-Script cDNA synthesis kit (BioRad). Non infected barley was used as a negative control and did not show any amplification. RT-qPCR were performed using 5 uL of KAPA SYBR FAST qPCR master mix (KAPA Biosystem), 4 µL of 1 : 20 diluted cDNA template and 500 nM final concentration of forward and reverse primers. Samples were run on a CFX96 Touch Real-Time PCR Detection System (BioRad). Actin, Fw 5' CCC AAT TTA CGA AGG TTT CTC TC 3' Rv 5' TCA GCG GTT GTG GAA AAA GT 3' was used as reference gene (Hacquard *et al*. 2013; Pennington *et al*. 2016). Thermocycling condition for all the probes were 95°C for 3 s and 63°C for 20 s. The primer efficiencies were determined for each probe through serial dilutions. Data were analysed using the CFX MANAGER 3.1 software (BioRad). Statistical analysis was done using R software.

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(6) Supplementary tables and figures

Table S1. List of the 5 differentially expressed genes from *Puccinia triticina* on Arina_*Lr34res* compared to Arina at high sporulation stage. a) List of the 4 up-regulated genes. b) One gene down-regulated.

a)

gene	fction	log FC
PTTG05637	hypothetical protein	-3.42
PTTG06615	hypothetical protein	-3.42
PTTG07513	hypothetical protein	-3.04
PTTG07625	hypothetical protein	-1.72

b)

gene	fction	log FC
PTTG03099	hypothetical protein	1.87

Table S2. List of the 4 differentially expressed genes from *Bgh* on barley_GLP_*Lr34res* compared to sib at 5 d.p.i a) List of the 2 up-regulated genes. b) List of the 2 down-regulated genes.

a)

gene	fction	log FC
EFBGRG00000000232	18S r	-3.17
bgh01363	CSEP	-1.8

b)

gene	fction	log FC
bgh03262	conserved predicted protein	1.6
bgh05191	opsin like protein	1.79

Table S3. List of the 38 differentially expressed genes from *Bgh* on barley_GLP_*Lr34* compared to sib, at 48 h.p.i., with their gene ontology function and the log fold change (log FC). a) 19 up-regulated genes b) 19 down-regulated genes. Arrows represent genes which were tested by RT-qPCR.

a)

gene	function	log FC	
bghG006495000001001	CSEP0466 putative effector protein	2.69	←
bghG002627000002001	Putative Bgh-specific protein	2.35	
bgh00541	Polyphosphoinositide phosphatase/Sac1	2.21	←
bghG001909000003001	Bgh-specific protein	2.05	←
bgh03071	Uncharacterized protein	2.02	←
bgh02919	Kinetochore protein mis13	1.94	
bghG006403000002001	Putative Bgh-specific protein	1.90	
bgh03184	Putative Bgh-specific protein	1.89	
bgh00359	C6 transcription factor (Mut3)	1.88	
bgh00172	Uncharacterized protein	1.80	
bgh00856	Histidine kinase	1.67	
bghG000610000001001	Sister chromatid cohesion protein Mis4	1.66	
bghG002591000002001	Putative effector protein	1.66	
bgh03504	MFS transporter	1.66	
bgh06119	Leucine-rich repeat protein	1.64	
bghG006466000001001	Putative effector protein	1.62	
bghG002234000001001	CSEP0346 putative effector protein	1.55	
bgh04923	Uncharacterized protein	1.52	
bgh02190	Bromodomain-containing protein	1.51	

b)

gene	function	log FC	
bghG003815000003001	Mitotic spindle biogenesis protein Spc19	-1.51	
bgh02262	CSEP0045 putative effector protein	-1.52	
bgh00585	Dolichyl pyrophosphate Man9GlcNAc2 alpha-1,3-glucosyltransferase activity/ALG6	-1.71	
bgh02591	Ubiquinone biosynthesis protein COQ4	-1.75	
bghG000243000001001	Uncharacterized protein	-1.76	
bgh03629	CSEP0123 putative effector protein	-1.77	
bghG008560000001001	CSEP0262 putative effector protein (CSEP0485 putative effector protein)	-1.78	
bgh06434	Arginine-tRNA-protein transferase	-1.86	
bghG002706000001001	\x09 O-methyl transferase B	-1.87	
bgh01382	SprT family metallopeptidase	-1.89	
bghG005930000001001	CSEP0442 putative effector protein	-1.89	
bgh04410	Uncharacterized protein	-1.94	
bgh01362	CSEP0027 putative effector protein	-1.97	
bghG005334000001001	Uncharacterized protein	-2.05	
bgh01572	Uncharacterized protein	-2.06	←
bgh02359	PAP2 domain-containing protein	-2.24	←
bgh02649	Uncharacterized protein	-2.31	←
bgh00631	Dihydrofolate reductase/DFR1	-2.90	
bgh03981	Putative mitochondrial nuclease	-3.16	

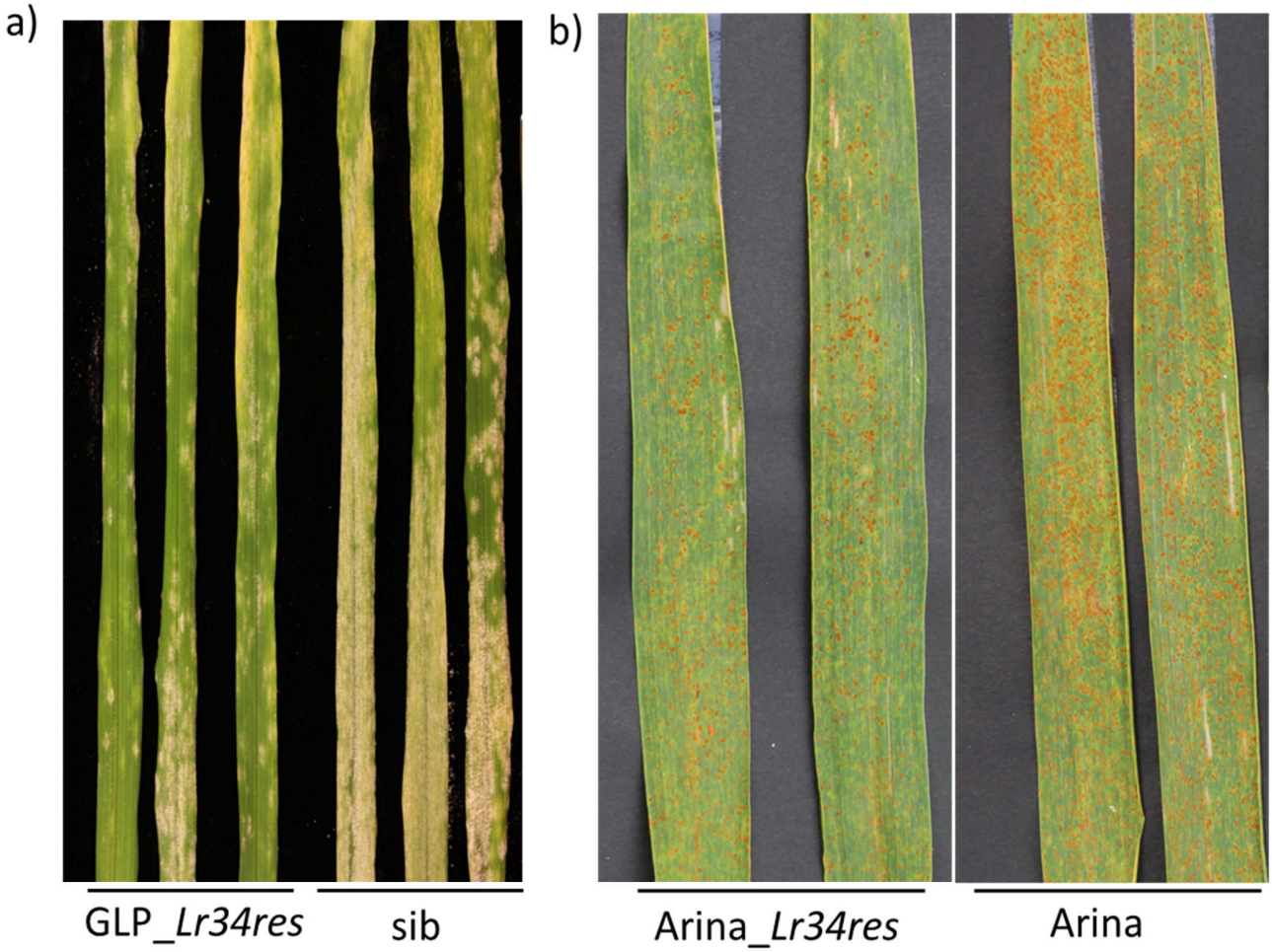

Figure S1. Macroscopical symptoms of a) 3-week-old GLP_*Lr34res* and its corresponding sib, infected with barley powdery mildew, 5 days after infection in greenhouse condition. b) Flag leaf of Arina_*Lr34res* and Arina infected with wheat leaf rust under field condition.

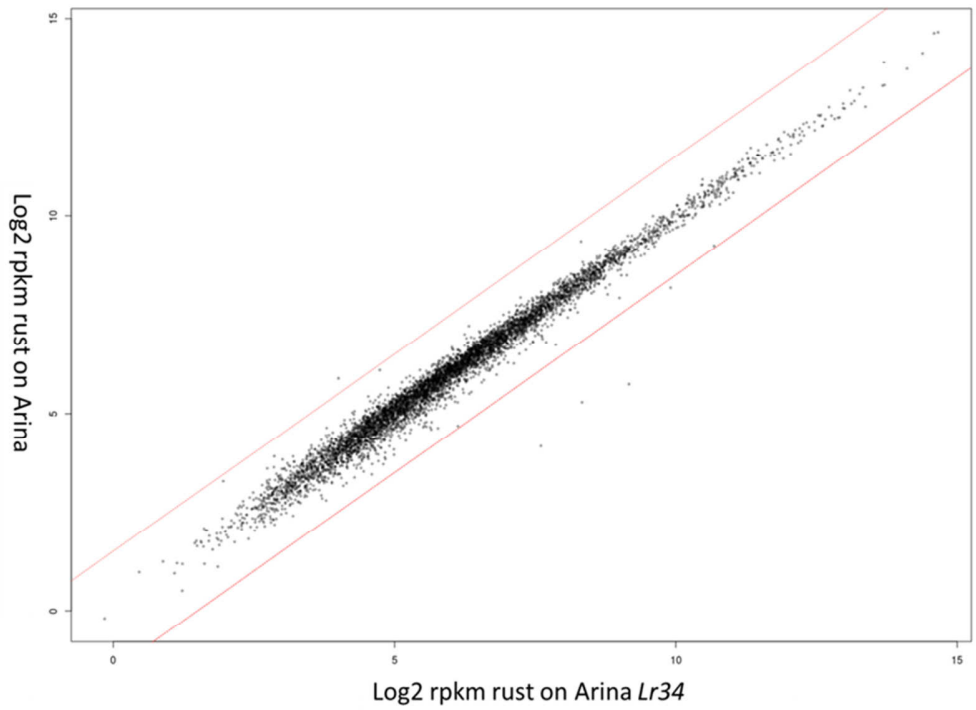

Figure S2. Plot of average changes in gene expression levels between *Puccinia triticina* growth on the two different hosts *Lr34res Arina* and Arina wheat at high sporulation stage from field infection. The values are shown in log2 of the number of reads per Kb per million mapped reads (rpkm). The two red lines represent the threshold of two log1.5 fold change in expression (equal to a 3-fold change). The general expression is very similar in the two hosts and there are no genes that are highly differentially regulated. Only 5 genes are significantly differently expressed in the two hosts (fdr corrected p-value < 0.01) with more than a 3-fold change.

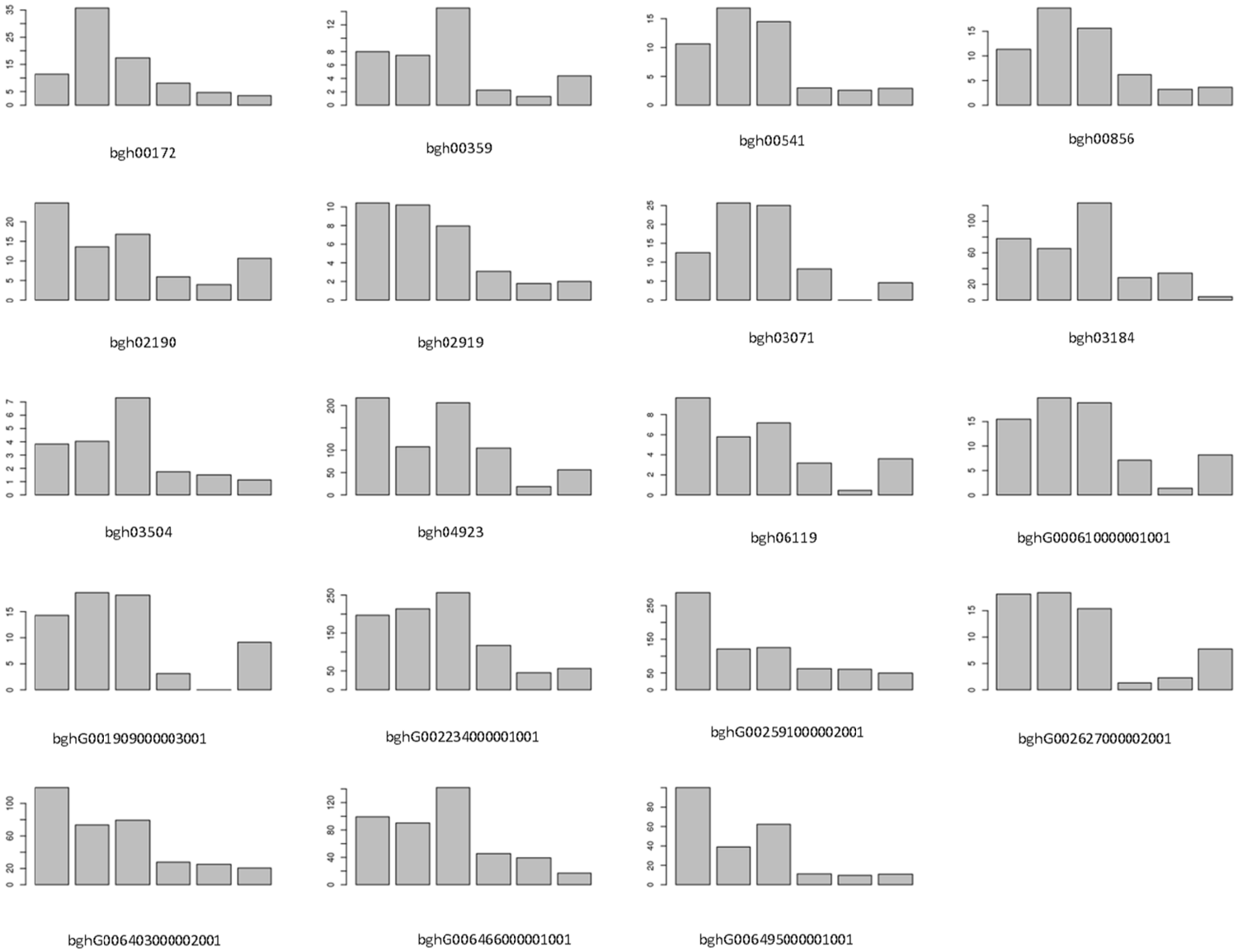

Figure S3. Expression level rpkm of the 19 up-regulated genes in *bgh* GLP_Lr34, 48 h.p.i. The three first columns represent Bgh on GLP_Lr34res and the three last *bgh* on sib.

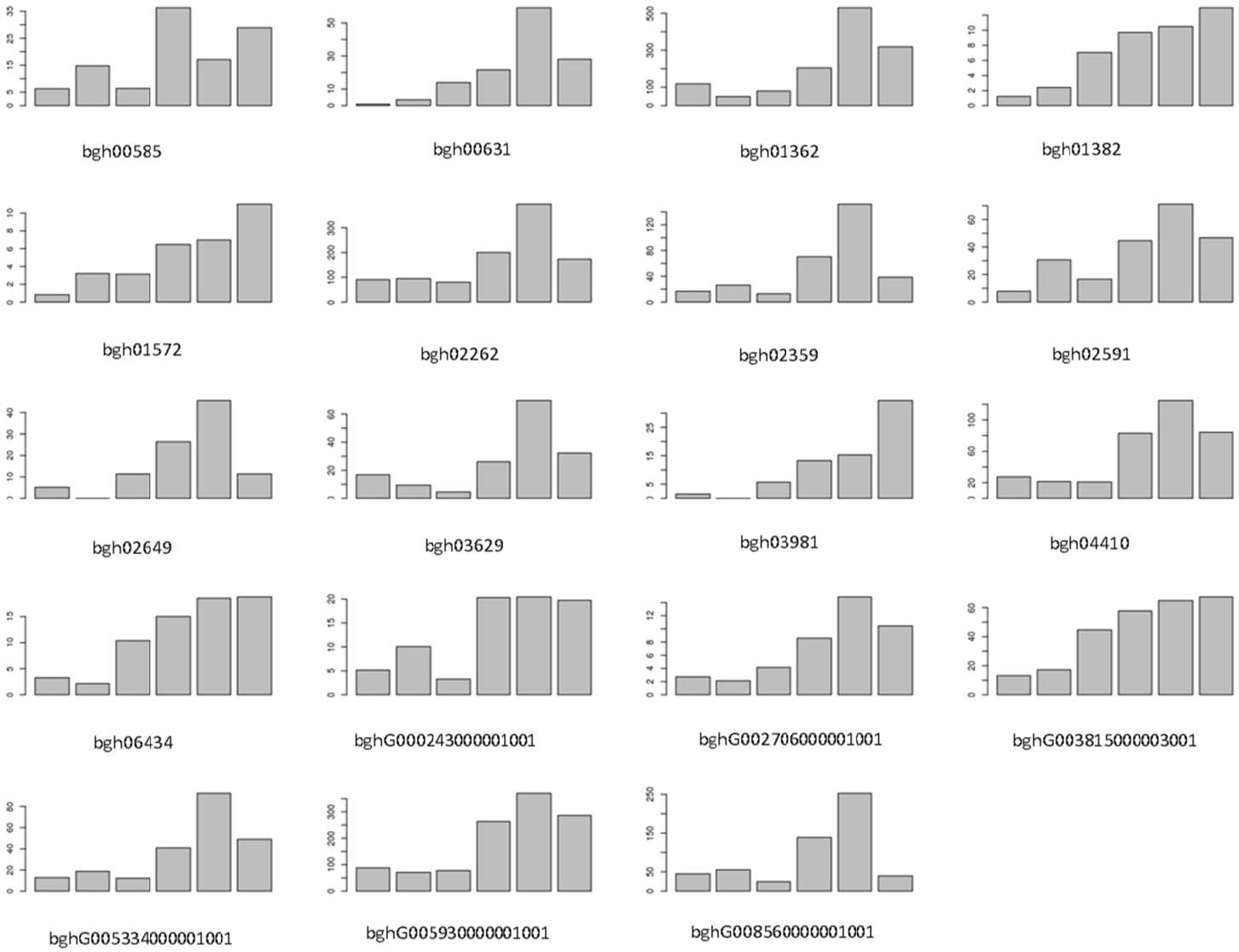

Figure S4. Expression level rpkm of the 19 down-regulated genes in *bgh* GLP_*Lr34*, 48 h.p.i. The three first columns represent *bgh* on GLP_*Lr34res* and the three last *bgh* on sib.

Table S4. One of the five independents simulations showing the number of DEGs according to the number of mapped reads.

		assigned reads		
	replicate	**GLP_*Lr34***	**sib**	**DEGs**
48 h.p.i total reads	1	1,269,940	670,369	**38**
	2	324,098	512,846	
	3	309,686	340,257	
5 d.p.i total reads	1	4,318,436	7,473,437	**4**
	2	2,114,538	9,799,292	
	3	4,997,687	7,685,958	
5% of the 5 d.p.i. total reads	1	215,483	373,248	**20**
	2	105,804	489,222	
	3	251,042	384,480	
10% of the 5 d.p.i total reads	1	431,508	746,588	**10**
	2	211,583	979,646	
	3	500,540	768,931	
20% of the 5 d.p.i total reads	1	864,027	1,464,032	**8**
	2	423,815	1,959,946	
	3	1,000,534	1,538,025	
50% of the 5 d.p.i total reads	1	2,160,129	3,737,862	**4**
	2	1,057,392	4,898,580	
	3	2,500,254	3,844,955	

Table S5. RT-qPCR primer sets designed for the gene expression experiments.

name	gene	sequence 5' 3'
541_F1	bgh00541	TGCAAGATGCATCGCTTTAC
541_R1		CATGTGCTGCATCGTAGCTT
3071_F1	bgh03071	ATGGGTTCTTTCCTGCTCCT
3071_R1		TTCCTTCACCTTCGAGTGCT
3001_F1	bghG001909000003001	ATGACCATCTGCGCCTATTC
3001_R1		GTCCTTAGCCAAATCGTCCA
1001_F2	bghG006495000001001	CTAACGTCGAGGCGAAAAAG
1001_R2		GACTCAGGGCTGTTTCCTTG
1572_F2	bgh01572	TTGTGCAATACGCCTCCATA
1572_R2		TTTGCCGTGAGAGTTGTGAG
2359_F2	bgh02359	TGTGGCATTTTTCTCCATCA
2359_R2		ACTCCTGCTCCAGGCAACTA
2649_F2	bgh02649	ACTCACATCGGCACACAAAA
2649-R2		AGTTTCGGGGGTGTCTCTCT

Chapter E

Conclusions and Outlook

The wheat gene *Lr34res* has been shown to be functional in major cereal species, where it confers broad spectrum resistance. In wheat and in barley the resistance has been shown against the respective wheat and barley leaf rust, stripe rust, stem rust and powdery mildew diseases (Risk *et al.* 2012; Risk *et al*. 2013). In rice, resistance was observed against rice blast (Krattinger *et al.* 2016) and in maize resistance against maize common rust and northern corn leaf blight (Sucher *et al.* 2016). These diseases are caused by the most important and devastating fungal pathogens in the world.

The *Lr34res* gene is unique to wheat and evolved from the susceptible version sometimes after 10 000 years ago, after wheat domestication (Krattinger *et al.* 2009; 2012). Rice carries an orthologous gene of *Lr34sus*, called *OsABCg50* but no resistant version has been identified so far (Krattinger *et al.* 2016). Domestication of rice and wheat occurred at the same time (Garris *et al.* 2005) but the gain of function of *Lr34sus* to *Lr34res* only occurred in wheat. It is possible that the orthologous gene of the resistant variant of *Lr34* exists in rice but that is a rare variant present only in a few genotypes which are not used in breeding. More than 40,000 rice varieties have been listed but only 100 are grown worldwide and only 10 are marketed and sold (Schenker 2012). Sequencing and analysis of the *OsABCg50* gene of these 40,000 cultivars could lead to the identification of *Lr34res* ortholog, but it is also possible that the characteristic polymorphisms in *Lr34res* only occurred in wheat. The deletion in the exon 11 has been shown to be sufficient, in rice and barley (Chauhan *et al*. 2015; unpublished data) to confer the leaf tip necrosis and resistant phenotype. The sequence changes (deletion and base change, as in *Lr34res*) introduced into *OsABCg50* did not result in leaf tip necrosis or resistance phenotype despite gene expression meaning that such a gain of function from *Lr34sus* might be unique to wheat. The absence of gain of function in the site-directed mutagenesis of the two residues in *OsABCg50* could be because of its expression level, due to its native promoter. Furthermore, the presence of *LR34res* in rice resulted in dramatic fitness cost in three of the four transgenic lines (Krattinger *et al.* 2016). This fitness cost has negative effects on the plant development and on the yield which represents a disadvantage for the plant. Under natural condition and selection pressure, this will lead to a negative selection and a loss of the gene associated with the negative phenotype. The wild relative of maize, *tripsacum* also carries an orthologous version of *Lr34sus* but no variant of *Lr34res*. Here also, the sequencing and of the orthologous gene of the 159 different CIMMYT accessions could

lead to an *Lr34res* variant identification. Constructs of site-directed mutagenesis (deletion and base change as in the *Lr34res*) in the *tripsacum* orthologous could be tested for gain of function.
In conclusion, the transgenic approach is for now the only possible way to obtain the *Lr34res* resistance in rice, in maize or in barley.

Lr34res has been used in wheat breeding programs for more than a century and has been shown to be durable with no pathogen adaptation observed so far. Results of our fungal transcriptomic analysis showed that *Lr34res* does not modulate gene expression of wheat leaf rust and barley powdery mildew. In other words, the pathogens do not modify gene expression in response to the presence of *Lr34res* which can explain its durability. Consequently, *Lr34res* should then also be durable in barley, rice and maize and would be a good source of resistance for cereal breeding.
Lr34res provides a partial resistance and it would still be necessary to combine it with additional resistance gene such as *Lr68* (leaf rust resistance) and *Sr2* (stripe rust resistance) to reduce the infection level (Silva *et al.* 2015). Having several resistance genes in combination and maintain a genetic diversity is important to prevent the risk of resistance breakdown by pathogens or resistance erosion due to a high selection pressure.
Growth penalties have been observed in barley and rice carrying the *Lr34res* gene. This growth reduction might be due to the high expression of the gene at the seedling stage, associated with a strong leaf tip necrosis (Risk *et al.* 2013; Krattinger *et al*. 2016). Nevertheless, among the different *Lr34res* transgenic rice lines one showed a development similar to the sister line but a resistance comparable to the other transgenic lines. This absence of strong negative effects of the gene has been correlated with a lower expression at the seedling stage (Krattinger *et al.* 2016). These results show that it is possible to modulate the effect of the gene according to the insertion site in the genome. By generating a large number of transformation events it should be possible to identify lines without strong fitness penalties. *Lr34res* maize lines all showed a development similar to the sister lines concerning plant height and fresh weight despite a strong expression of *Lr34res* at the seedling stage (Sucher *et al*. 2016). The exact fitness cost would need to be assessed in a field experiment in order to have the full information about the effect of *Lr34res* in rice and in maize.
The fact that no obvious growth penalty was observed in *Lr34res* maize in the greenhouse contrasts with the results in rice and barley and is possibly caused by different metabolisms in

different species. One of the main differences between rice and maize is their photosynthesis process modes. Rice uses a C3 pathway compared to maize which has a C4 one. C4 and C3 photosynthesis differ in the efficiency with which they consume water and nitrogen (Wang *et al*. 2014). Under low stomatal conductance, the C4 plants are still able to maintain high photosynthetic rate compared to the C3 plants. Stomatal conductance has been shown to be lower in the transgenic rice lines and is modulated by the ABA concentration. ABA regulates many biologically important processes including stomatal closure and drought tolerance which are in direct connection with the photosynthesis process. Indeed, in 2007, Hulbert *et al.* showed by a wheat transcriptomics analysis that most of the up-regulated genes between *Lr34res* and wild type plants were associated with ABA inducibility and drought stress. Recently, Krattinger *et al*. (unpublished) showed that *Lr34res* modulate the abscisic acid (ABA) fluxes in the plant.

Crop protection from disease is a real challenge to reduce yield losses and satisfy the demand of food from an increasing world population. The use of fungicides is expensive, time consuming and can be harmful for the environment and also the population living in the neighborhood (Halwachs *et al.* 2013; Damalas and Eleftherohorinos 2011). Plants with genetic resistance would be a good alternative to reduce or even stop fungicide treatments. Interspecies gene transfer is a very efficient method to move resistance between species and increases the resistance spectrum. As example, the rice gene *Xa21* (coding for a receptor-like kinase) was functionally transferred into banana where it conferred resistance against Xanthomonas wilt (Tripathi *et al.* 2014). Results presented in this thesis validate the functional transfer of the *Lr34res* wheat gene in cereals of interest and showed that this gene would be a good tool for plant breeding in order to reduce yield losses due to fungal pathogens. The next step would be to transform *Lr34res* into elite varieties such as the rice IR36 or IR64 (Balini *et al*. 2007; Wu *et al.* 2005) or the maize CG00526 (Que *et al.* 2014) and to assess their resistance and yield levels under different field conditions.

During this PhD thesis, new *Lr34res* plant material was developed in order to have a better understanding on the *Lr34res* resistance mechanism. The transgenic *Lr34res* expressing rice and maize plants can be used as very useful tools to study the *Lr34res* resistance mechanism. Rice and maize both have a diploid genome and are easier to study than the hexaploid wheat. Furthermore, the rice genome is 40 times smaller (420 Mb) than the wheat ones (16 Gb) and is fully sequenced and well annotated (International Rice Genome Sequencing Project 2005).

Lr34res has been shown to modulate the ABA fluxes in the plant (Krattinger *et al*. unpublished) and to confirm these results one approach would be to study *Lr34res* ABA deficient plants. The reduction or lack of the ABA substrate in the plant might lead to a LR34res transporter activity reduction which could result in absence of LTN and resistance. ABA is a hormone essential for the plant development since it plays a major role for the seed maturation and germination but also for the biotic and abiotic stress responses (del Carmen Rodríguez-Gacio *et al*. 2009). ABA deficient mutant are, in most of the cases albinos or pale and non-viable seedlings, but some mutants are available, including the viviparous ABA deficient mutant. In maize, the different viviparous *vp12*, *vp1* and *vp14* heterozygous parents could be used for crosses with the *Lr34res* homozygous transgenic maize (Maluf *et al.* 1997; McCarty *et al.* 1989; Tan 1997). The segregating population would be analyzed on the DNA level for the presence of the *Lr34res* gene and the reduction of the ABA concentration will be measured by ELISA essay. Phenotypic observation of the presence/absence of the LTN and infection test could be performed to assess the resistance.

A second approach to validate the substrate pathway would be a mutagenesis which would induce point mutation in genes necessary for *Lr34res* function. During this thesis, an ethylmethansulfonat (EMS) experiment was performed on ~2,500 *Lr34res* transgenic rice seeds. A highly expressing line was chosen because of its clear and early LTN phenotype which is a good indicator of *Lr34res* expression in the plant (Krattinger *et al.* 2016). Two different EMS concentrations has been chosen, 0.6% and 0.8% and among the 2,500 mutated M0 plants, 1,250 gave M1 seeds, which were individually harvested. Between 8 to 12 M1 seeds per line were screened for the absence of LTN and also for albinos phenotype. The observed albinos rate, which is an indicator for successful treatment, was 9.6% for the 0.8% EMS and 4.3% for the 0.6%., similar to the results of Till *et al.* (2007) who showed a 8% frequency of albinos plants for a rice populations that had a suboptimal mutation rate.

Among the ~15,000 M1 screened plants, no LTN mutant of interest was identified. Since the *Lr34res* rice line used for the experiment was a single insertion copy, mutants in the *Lr34res* gene was expected. The absence of *Lr34res* mutants as well as mutants of interest can be explained by the low number of mutated seeds. The mutation density in rice is one for every 300 kb, compared to wheat where the density is 1/38 kb (Rawat *et al.* 2012), meaning that the number of mutated rice seeds would need to be increased of about 10 times in order to saturate the entire genome with point mutations.

Thus, in order to be able to identify a mutant in a gene of interest, a mutagenesis would have to be performed on ~20,000 rice seeds in order to analyze at least 10,000 M1 lines. As the rice plants are transgenic, the field propagation would be difficult due to the GMO regulations on one hand but also because this line has never been tested under natural condition and we don't know how it will behave concerning growth habits yield. The M1 screen should also be performed in the field, due to the large number of plants to screen. 10 M2 plants for each M1, meaning 100,000 plants, would have to be screened in order to identify mutants of interest.

It would be necessary to find collaborators (preferentially in Asia: China, Japan or Phillipines) who could provide field space for this huge number of plants. Mutants obtained could also be due to the knockout of the *Lr34res* gene itself, hence its integrity will need to be confirmed by full length sequencing. Only mutants with an intact version of *Lr34res* will be of interest to identify genes involved in the *Lr34* resistance mechanism. Point mutations can be identified by complete genome sequencing of the mutants and their corresponding wild type, as described in the MutMap+ method (Fekih *et al.* 2013). The (single nucleotide polymorphism) SNP due the EMS point mutation will be detected by sequenced alignment. Thanks to a good annotation of the rice genome it will be possible to identify the mutated gene and have indication about its function in the plant and have a better indication of the *Lr34res* pathway.

An additional unanswered question relates to the cellular and sub-cellular localization of the LR34 protein which would provide information about the resistance mechanism of *Lr34res*. For the cellular localization, an *Lr34res* gee construct with the genomic sequence under the wheat native promoter fused with either the GFP reporter protein (green fluorescent protein) or a (Hemagglutinin) HA-tag was transformed into barley plants. The expression of the LR34-GFP was assessed by western blot analysis but no signal could be detected. The large size of the GFP-tag (238 amino acid) (Kanda *et al.* 1998) possibly interfered with the localization and resulted in an absence of signal. Therefore the use of the HA-protein, which only has an additional epitope of 9 amino acids (Kaltwasser *et al.* 2002), would be a good alternative to localize LR34 in barley. Next steps will be to perform the localization with the barley *Lr34res* Ha-tagged leaves.

An additional method for the cellular localization of LR34 would be by the agro-infiltration of the LR34-GFP and LR34-HA genomic constructs under the 35S promoter in Nicotiana benthamiana. Expression after infiltration has already been confirmed, but not the localization of the protein.

The sub-cellular localization can be performed based on protoplast transformation with the LR34res-HAtagged or GFP-tagged constructs, using the polyethyleneglycol (PEG) method, as described in Lazzeri *et al.* 1991. By the experiments as described above, critical information for understanding the durability and the large action spectrum of *Lr34res* could be obtained.

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